ABBERANT ANXIETY IN SCHIZOPHRENIA: A POTENTIAL TARGET FOR EARLY INTERVENTION

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Schizophrenia, as a neurodevelopmental disorder, arises from interaction of genetic predisposition and early life risk factors that lead to the onset of psychosis in late adolescence or early adulthood. Several studies on adolescents at high risk for schizophrenia have found higher level of anxiety associated with emergence of psychosis later in life. Thus alleviating this heightened anxiety might be an effective way to prevent the transition to psychosis.

Rats exposed during gestational day (GD) 17 to the mitotoxin methyl azoxymethanol acetate (MAM) exhibit behavioral, pharmacological, and anatomical characteristics consistent with an animal model of schizophrenia. These rats exhibit increases in dopamine neuron population activity (i.e. the proportion of spontaneously active DA neurons) and the enhanced locomotor response to amphetamine, which correlate with the psychosis. This hyperresponsivity of dopamine system could be driven by abnormal activity of the hippocampus, resulted from loss of parvalbumin interneurons.

In this study, we observed in peripubertal MAM rats higher level of anxiety as tested in elevated plus maze and higher firing rate of basolateral amygdala neurons, which persisted into adulthood. In addition, peripubertal MAM rats demonstrated increased conditioned stimuli-induced freezing in a standard fear conditioning paradigm. Although adult MAM rats did not

show significant difference on such freezing behaviors, they had significantly more increase in power of theta oscillations in response to these conditioned stimuli.

To alleviate anxiety, a widely used anti-anxiety drug diazepam was administered to rats during peripubertal period. Peripubertal administration of diazepam was found to prevent the increase in dopamine neuron activity and attenuate the behavioral hyper-responsivity to amphetamine in MAM rats. These effects of peripubertal administration of diazepam could be partially due to its attenuation of loss of parvalbumin positive neurons in the ventral subiculum of the hippocampus in these rats. In addition, peripubertal administration of diazepam has a persistent effect to reduce anxiety-like behaviors in the elevated plus maze, reduce conditioned stimuli induced freezing behaviors, and normalize hyperactivity of basolateral amygdala in MAM rats.

Altogether, these results suggest that pathophysiological factors leading to the onset of psychosis in early adulthood may be circumvented by controlling anxiety during adolescence.

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1.0 INTRODUCTION

1.1 SCHIZOPHRENIA

Schizophrenia is a debilitating mental disorder that affects approximately 1 % of the population worldwide (Weinberger, 1987; Lieberman et al., 2001). Symptoms demonstrated in patients with schizophrenia can be divided into three categories: positive symptoms, negative symptoms, and cognitive symptoms. Positive symptoms are psychotic symptoms including hallucinations, delusions, and unusual thinkings; negative symptoms include emotional symptoms like affective flattening and loss of pleasure, as well as loss of speech, loss of motivation and social withdrawal; and cognitive symptoms include impaired working memory, behavioral inflexibility, attention deficits, etc. Based on Diagnostic and Statistical Manual of Mental Disorders (DSM) IV, the most important criteria for schizophrenia diagnosis are based on psychotic symptoms. Declining in emotional and cognitive functions in schizophrenia patients could begin early in childhood but psychotic symptoms usually emerge in late adolescence and early adulthood.

Schizophrenia is widely believed to be a neurodevelopment disorder. Schizophrenia is heritable, having a first-degree relative increases the risk of have schizophrenia. Indeed, many candidate genes have been identified to be related with schizophrenia, e.g. DISC1, NRG1 (Jaaro-Peled, 2009; Mei and Xiong, 2008). However, considering the onset of psychosis until late adolescence, genetic predisposition alone is insufficient to account for this delayed onset.

Instead, a two-hit model of schizophrenia was proposed, in which genetic predisposition or prenatal insults disrupt early brain development, render individuals susceptible, and the "second-hit" risk factors lead to the onset of psychosis in late adolescence and early adulthood (Maynard et al, 2001). Several risk factors have been identified as related to schizophrenia, including maternal stress, maternal infection and nutritional deficiency, childhood trauma, living in urban areas, cannabis use (van Os et al., 2010). Stress seems to be the factor that trigger to onset of psychosis, as reported by a number of studies that patients experienced more major life events preceding the onset of psychosis (Phillips et al, 2007).

1.1.1 Pathophysiology of schizophrenia

Increased activity in the dopamine system is thought to underlie psychotic symptoms of schizophrenia. As measured by D2 receptor radioligand displacement, schizophrenia patients have increased basal extracellular dopamine, and psychostimulant-induced mesolimbic dopamine release is associated with exacerbation of active psychosis (Laruelle and Abi-Dargham, 1999; Abi-Dargham et al, 2000). The involvement of dopamine system is also supported by actions of most antipsychotics as their primary actions are dopamine D2 receptor antagonism. However, dopamine system itself may not be the site of pathophysiology, instead, it is the dysregulation of dopamine system that may underlie the pathophysiology of schizophrenia.

Alterations in many brain regions are related with pathophysiology of schizophrenia. Postmortem studies from PFC of schizophrenia patients demonstrated deficits in GABAergic system especially in parvalbumin positive interneurons as well as altered dopamine signaling (Volk and Lewis, 2002; Winterer and Weinberger, 2004). Impaired cognitive functions should be related with deficits in the PFC of schizophrenia patients.

The hippocampus is also widely studied in schizophrenia patients. One of the most robust findings is reduced hippocampal volume (~5%) both in MRI and postmortem studies (Bogerts 1997; Heckers and Konradi 2002); smaller neuron size has also been reported (Benes et al, 1991). Altered hippocampal expression of protein involved in glutamatergic, GABAergic, dopaminergic signaling as well as synaptic structure and functions have all been reported (Knable et al., 2004; Torrey et al., 2005). Enhanced hippocampal activity at rest have been reported in schizophrenia patients, which is correlated with positive symptom severity (Heckers et al., 1998; Malaspina et al., 1999; Medoff et al., 2001; Lahti et al., 2006; Schobel et al., 2009); and failure to recruit the hippocampus was observed on schizophrenia patients on memory-related tasks (McCarley 1993; Heckers et al, 1998; Weiss et al, 2003, 2004). Loss of parvalbumin positive interneuron was also observed in the hippocampus (Zhang and Reynolds, 2002).

Moreover, the hippocampus is critical for regulation of dopamine system activity (Grace et al, 2007). In schizophrenia, an increase in amphetamine-induced DA release in schizophrenia that correlated with exacerbation of psychosis (Laruelle et al, 1999). Psychosis was also correlated with hyperactivity in the limbic hippocampus (Malaspina et al, 1999; Medoff et al, 2001; Molina et al, 2003). And in ultra-high-risk individual alterations in hippocampal glutamate levels occurred in concert with increased presynaptic indices of dopaminergic function (ie, fluorodopa uptake; Schobel et al, 2009; Stone et al, 2010; Howes et al, 2011). Taken together, these data support a model in which hippocampal hyperactivity leads to increases in DA neuron population activity, rendering the system hyperresponsive to stimuli (Lodge and Grace, 2007).

1.1.2 Treatment for schizophrenia

Discovery of antipsychotics are revolution for schizophrenia treatment. The first generation antipsychotics (typical antipsychotics, e.g. haloperidol) are primarily as dopamine D2 receptor antagonists, suppressing dopamine activities in the mesolimbic pathway, thus alleviating psychotic symptoms. However, treatment with typical antipsychotics is often associated with extrapyramidal side effects (EPS) related with reduction of dopamine in the nigrostriatal pathway and other side effects. The second generation antipsychotics (atypical antipsychotics, e.g. clozapine, risperidone, aripiprazole) have affinities not only to dopamine D2 receptors but also to 5-TH2A and other serotonin receptors. The major advantage of atypical antipsychotics is the lower incidence of EPS. But they still induce side effects including weight gain and disruptions in glucose and lipid metabolism. In addition, although both typical and atypical antipsychotics effectively reduce psychotic symptoms, they have very few therapeutic effects on negative and cognitive symptoms (Miyamoto et al, 2012).

Antipsychotics exert therapeutic actions during the first days of administration, maximal antipsychotic actions require weeks to develop, and patients do not develop tolerance to its actions (Kapur and Seeman, 2001), all of which cannot be explained by its dopamine D2 receptor antagonism. Instead, one mechanism that could explain the course of its action and lack of tolerance is depolarization block, in which actions of antipsychotics induce an excitation-mediated inactivation of dopamine neuron firing. Although effectively suppressing psychosis, antipsychotics does not seem to reverse its pathophysiology, instead, it induces an offsetting pathological condition (Grace et al, 1997).

For schizophrenia patients, early treatment is vital. Duration of untreated schizophrenia correlates with a worsened prognosis in patients (Hill et al, 2012). And an even more effective

early treatment is prevention. Before onset of psychosis, patients already exhibit emotional and cognitive deficits, and in the prodromal phase, attenuated psychotic symptoms. Many biological and social changes underpinning the development of schizophrenia may already be active in the pre-psychotic or prodromal phase. Indeed, abnormal cerebral blood volume increases and hypermetabolism were observed in the hippocampus of at-risk human subjects, indicative of a hyperactivity of hippocampus (Schobel et al, 2009; Schobel et al, 2013). In addition, the pre-psychotic phase may be the most sensitive part of the 'critical period' for preventive efforts (Phillips et al, 2002).

It is very difficult to diagnose schizophrenia early. However, a lot of efforts have been made for early diagnosis by studies focusing on subjects at risk for schizophrenia. Individuals that are at risk for schizophrenia can be identified based on genetic background and family history (Sullivan et al, 2003; Straub and Weinberger, 2006). Structured interviews for evaluating psychosis risk such as the Structured Interview for Psychosis Risk Syndromes (Miller et al, 2003) and the Comprehensive Assessment of At-Risk Mental States (Yung et al, 2005) also contribute substantially toward creating a reliable and valid system for identifying risk before psychosis onset. Researchers have identified some symptoms that correlate with later onset of psychosis. Increased anxiety and impaired tolerance to normal stress in childhood and adolescence are some of these factors that are predictive of onset of psychosis later in life (Owens et al, 2005; Yung et al, 2005; Corcoran et al, 2012; Devylder et al, 2013).

1.1.3 Stress and schizophrenia

Stress is one of the risk factors related with development of schizophrenia (Walker and Diforio, 1997; Corcoran et al, 2003; Thompson et al, 2004). Several studies reported that patients

experienced more major life events preceding the onset of psychosis (Phillips et al, 2007). However, these studies are mostly retrospective. Therefore, they cannot rule out the possibility that patients with schizophrenia tend to perceive more life events as stressful, which normal subjects would just perceive as neutral or mild. Indeed, some studies have reported lack of relationship between stressful life events and psychosis, but instead, a relationship between impaired tolerance to stress/ anxiety and psychosis. (Owens et al, 2005; Yung et al, 2005; Corcoran et al, 2012; DeVylder et al, 2013).

Owens et al (2005) reported that, in adolescents at high genetic risk for schizophrenia, those who showed a higher premorbid anxiety response to stress tended to be the same individuals who eventually converted to schizophrenia later in life. In addition, impaired tolerance to stress is associated with a range of prodromal symptoms and poor function over time (Devylder et al., 2013) and may be predictive of the development of psychosis (Yung et al, 2005). Anxiety is an emotional response to stress, and correlation between heightened anxiety and impaired tolerance to stress was also observed (Devylder et al., 2013).

As for the biological evidence for hyperresponsivity to stress, some studies (Walker et al., 2001, 2010) showed that baseline cortisol levels in adolescents with schizotypal symptoms predict severity of their schizotypal symptoms later in life. In addition, there were reports of an enlarged hypothalamus (Goldstein et al., 2007) and pituitary gland (Garner et al., 2005; Mondelli et al., 2008; Habets et al., 2012) in schizophrenia patients and nonpsychotic relatives, which is consistent with sustained stress. Moreover, a larger baseline pituitary volume was a significant predictor of future transition to psychosis within the ultra-high risk group (Garner et al., 2005).

1.1.4 Anxiety and schizophrenia

In addition to the correlation between premorbid anxiety and onset of psychosis (Jonhstone et al, 2005; Owens et al, 2005; Corcoran et al, 2012; DeVylder et al, 2013), anxiety is highly prevalent throughout the course of schizophrenia.

Anxiety disorders include generalized anxiety disorder, social anxiety disorder, panic disorder, post-traumatic stress disorder, obsessive compulsive disorder, etc. These disorders share the feature of exaggerated fear and anxiety. Fear is the emotional response to acute threat, whereas in anxiety the threat is thought to be more chronic and diffuse. Anxiety could also be seen as anticipation of future threat, which usually associates with cautious and avoidant behaviors.

Comorbidity of anxiety disorders with schizophrenia is about 38.3%(Buckley et al., 2009, Achim et al., 2011). In addition, some evidence suggests that schizophrenia patients with comorbid anxiety disorders show a decline in their subjective quality of life (Braga et a., 2005). Therefore, anxiety in schizophrenia patients is an extra burden for their functioning and quality of life, which should be considered in their treatment. In addition, in more than 50% of schizophrenia patients, anxiety disorders preceded the onset of psychosis (Pokos and Castle, 2006).

1.2 ANIMAL MODELS OF SCHIZOPHRENIA

Schizophrenia is a disorder unique to humans. Many symptoms of schizophrenia cannot be examined in animals, e.g. hallucinations, delusions, disorganized thought, loss of speech.

However, efforts in generating animal models of schizophrenia are priceless in studying mechanisms underlying the pathophysiology of schizophrenia, development trajectory, and potential treatment. Various animal models for schizophrenia have been developed. Pharmacological models (e.g. administration of PCP, Jentsch ad Roth, 1999) produces immediately behaviors related with psychosis and cognitive impairment; however, it does not recapitulate the development course of the disease. Genetic models are based on findings of candidate genes associated with risk of schizophrenia (e.g. DISC1, NRG1, Jaaro-Peled, 2009; Mei and Xiong, 2008), which better mimic genetic component of schizophrenia etiology. However, mutation of a single gene is not responsible for schizophrenia and thus cannot adequately replicate symptoms of schizophrenia. Manipulation on a cluster of genes seems to be a better way to generate genetic model of schizophrenia (22q11.2 deletion, Sigurdsson et al, 2015). Neurodevelopmental models of schizophrenia (e.g. maternal immune activation, neonatal ventral hippocampal lesion, MAM-E17, Lipska, 2004; Grace et al, 1998) disrupt early brain development and lead to behaviors correlated with psychosis to emerge in adulthood. These models have the unique advantage to study development of schizophrenia.

1.2.1 MAM-E17 developmental disruption model of schizophrenia

MAM injection on embryonic day 17 (E17) disrupts brain development and leads to many alterations in brain morphology, functions, and behaviors related with schizophrenia (Lodge et al., 2009, Modinos et al., 2015). Methyl azoxymethanol acetate (MAM) is a neurospecific anti-mitotic agent that prevents cell division for 12 to 24 hours after its injection. Therefore, administration of MAM would have a major impact on brain regions that are actively developing during the time of its action. On embryonic day 17 (E17) is the peak proliferation of the

hippocampus (Bayer, 1980). Indeed, disruption of cell division in the hippocampus by MAM injection on E17 was supported by decrease of BrdU-positive cells in this region (Hoareau et al, 2006).

The timing of MAM injection is critical. Various time of injection were used in different studies, ranging from E9 (Jongen-Relo et al, 2004; Talamini et al, 2000) to postnatal period (Lu et al, 2000). Disruptions on brain morphology and behaviors produced by MAM administration on or before E15 are too widespread and nonspecific to schizophrenia (Gourevitch et al, 2004; Moore et al, 2006). Brain regions that are important in pathophysiology of schizophrenia, prefrontal and temporal cortices (Akbarian et al, 1993a; Akbarian et al, 1993b) are actively developing during E17. Indeed, administration of MAM on E17 leads to much less intense specific damage on these brain regions, as well as altered behaviors known to be impaired in schizophrenia patients (Flagstad et al, 2004, 2005; Gourevitch et al, 2004; Moore et al, 2006).

Rats with MAM model of schizophrenia exhibit deficits in tasks related with working memory, behavioral flexibility, attention, sensory motor gating, and social interactions (for review, see Lodge and Grace, 2009). These rats show enhanced locomotor response to amphetamine challenge, which occurs only in adulthood, consistent with the onset of psychosis (Moore et al., 2006). Hyperactivity of the dopamine system is thought to underlie hyper locomotion in response to amphetamine, which is driven by hippocampal hyperactivity in these MAM- treated rats (Lodge and Grace, 2007). Furthremore, a loss of parvalbumin positive interneurons (Penschuck et al., 2006, Lodge et al., 2009) is thought to contribute to this hippocampal hyperactivity, which is consistent with postmortem observations in schizophrenia brains (Zhang and Reynolds, 2002). Parvalbumin interneurons are responsible for generation of gamma oscillations (Sohal et al, 2009). Therefore, impaired gamma oscillation in the

hippocampus, measured as response to a conditioned tone in a latent inhibition paradigm, might also be related with loss of parvalbumin interneurons (Lodge et al., 2009).

Moreover, MAM rats are more anxious and more responsive to stress exposure. Researchers have observed a blunted yet non-adaptive corticosterone level, more freezing and more ultrasonic vocalizations in foot shock exposure in juvenile and adolescent MAM rats (Zimmerman et al., 2013). These findings are consistent with those from human studies about aberrant cortisol level, stress intolerance and a heightened anxiety level (Owens et al, 2005; Yung et al, 2005; Walker et al, 2010; Corcoran et al, 2012; Devylder et al, 2013).

1.2.2 Use of animal model of schizophrenia to study developmental trajectory and early interventions

Neurodevelopment models of schizophrenia have the unique advantage of recapitulating the development of schizophrenia. Therefore, it is a great tool to study developmental trajectory and possible early interventions for schizophrenia. Studies in MAM model of schizophrenia found that impairment in parvalbumin expression in MAM rats begins long before puberty (PD25) and lasts to adulthood in which MAM rats exhibit a progressive loss of parvalbumin across development (Chen et al, 2014; Gil and Grace, 2014). Antioxidant agents administering to juvenile rats with neonatal ventral hippocampal lesions effectively protect the loss of parvalbumin and restore functions of PFC (Cabungcal et al., 2014). In the maternal immune activation model of schizophrenia, administration of antipsychotic drugs to adolescent rats has been shown to circumvent the emergence of several correlates of schizophrenia in adult rats (Pointkewitz et al, 2011, 2012).

1.3 THE HIPPOCAMPUS

The hippocampus is located in the temporal lobe, which is essential for formation of episodic memories. The functions of hippocampus are different as for the ventral and dorsal parts, in which dorsal part is more responsible for spatial information processing whereas the ventral part processes contextual information, and is related with anxiety (Bannerman et al, 2003). The hippocampus comprises several subregions including dentate gyrus, CA3, CA1 and the subiculum. The subiculum is the major output structure of the hippocampus. Ventral subiculum (vSub) of the hippocampus is the target focus in our studies. It is continuous with CA1 area and is marked with an abrupt widening of the pyramidal cells layers. It is composed of three layers: a molecular layer, continuous with that of CA1; a pyramidal layer, wider and more diffuse than that in CA1, and a polymorphic layer (O'mara et al, 2001). The vSub comprises of glutamatergic pyramidal cells and GABAergic local interneurons. Interneurons that express parvalbumin are of particular interest because they are fast spiking interneurons that control gamma oscillations (Sohal et al, 2009) as well as contribute to theta oscillations (Amilhon et al., 2015).

Ventral hippocampus is critical in regulation of VTA dopamine neuron population activity. Unlike regular pacemaking in vitro, half of dopamine cells in vivo are silent under inhibitory projection from ventral palladium (VP) or other brain regions. The other half that are spontaneously firing are either firing irregularly or bursting, under the influence of excitatory or inhibitory information sent by other brain regions. The proportion of dopamine neurons that are spontaneously firing is controlled by the ventral hippocampus via projections to the nucleus accumbens (NAc) (Floresco et al, 2001). Excitation of the hippocampus leads to increased activity of NAc, inhibition on VP thus potentiated, which removes the inhibition on VTA dopamine neurons provided by VP (Floresco et al, 2001; Grace et al, 2007).

Alterations in the hippocampus are the most robust findings in schizophrenia patients, both morphologically and functionally. And similar alterations were observed in the MAM-E17 developmental disruption model of schizophrenia. The peak of cell proliferation in prenatal development of the hippocampus is E16-18 (Bayer, 1980), which makes the hippocampus most vulnerable to administration of the mitotoxin MAM on E17. Loss of parvalbumin interneuron in the ventral hippocampus leads to disinhibition of hippocampal activity, which in turn result in increase in VTA dopamine neuron population activity in MAM-treated rats, underlying amphetamine-induced hyperlocomotion, the behavioral correlate of psychosis (Flagstad et al, 2004; Lodge and Grace, 2007; Lodge et al, 2009).

1.4 THE AMYGDALA

The amygdala is located in the temporal lobe, ventromedial to the striatum, and anterior to the ventral hippocampus. It is part of the limbic system, which is intensively studied for its critical functions in fear and anxiety.

1.4.1 Anatomy and physiology

The amygdala is a complex comprised of many nuclei that are diverse in cytoarchitecture, histochemistry, connections with other regions, and functions. The basolateral amygdala (Lister et al.) is the focus in our studies, which comprises the lateral, basolateral, and basomedial nuclei (McDonald, 1998; Sah et al, 2003). These three nuclei can be further divided into more subdivisions, but functionally similar, they are grouped as the basolateral amygdaloid complex

(Lister et al.). These nuclei within BLA are extensively interconnected. The predominate type of neurons in BLA are large pyramidal neurons that are glutamatergic projection neurons, with long duration of action potentials, and low spontaneous firing. A very small proportion of projections neurons in the BLA are of stellate shape, with a relatively shorter action potential duration and higher firing rate (Rainnie et al, 1993). The BLA also contains 20-30% of GABAergic interneurons, which could be further divided into subtypes that express parvalbumin (PV), calbidin, somatostatin, cholecystokinin, etc. (McDonald, 1985). PV interneurons constitute about half of the interneuron population in the BLA and a lot of them co-express calbindin (McDonald et al, 2001). GABAergic interneurons send extensive inhibitory projections locally, and provide a potential local inhibition to projection neurons in the BLA.

The BLA is extensively interconnected with other nuclei within the amygdala, e.g. the projection to the central nucleus of the amygdala (CeA) is the major information flow within the amygdala and is critical in fear learning and other functions (Pitkänen et al, 1997; Maren and Quirk, 2004). Through projections to CeA and the bed nucleus of the stria terminals (BNST, also extended amygdala), BLA can influence functions brainstem structures and autonomic response. BLA receives sensory information of all modalities from cortices and thalamus, working as a hub linking brain regions that process sensory information and brain regions that are responsible for emotional response and actions. The projection from cortical areas to BLA is highly reciprocal. BLA and cortical areas that are responsible for cognitive functions are interconnected too, e.g. prefrontal cortex (PFC) has reciprocal connections with BLA, through which PFC regulates fear learning and extinction (McDonald, 1998; Maren and Quirk, 2004).

The hippocampus is intensively interconnected with the BLA. The BLA receives projections from CA1 and subiculum of the hippocampus and sends substantial projections to the

hippocampus, including various subregions of the hippocampus- CA3, CA1 and subiculum (McDonald, 1998; Pikkarainen et al, 1999).

1.4.2 Involvement in fear and anxiety

The neural circuitry of fear conditioning has been extensively studied, in which BLA plays a central role in fear acquisition and expression (Fanselow and LeDoux, 1999; Maren and Quirk, 2004; LeDoux, 2007). Fear conditioning increases firing of individual BLA neurons (Paré and Collins, 2000; Rosenkranz and Grace, 2002; Goossens et al, 2003). Moreover, fear conditioning increases synchrony in the BLA, in particular at the theta frequency (Sneidenbechet et al, 2003; Pelletier JG and Paré, 2004). Synchrony between BLA and other brain regions including the hippocampus and PFC also increases after fear conditioning (Sneidenbechet et al, 2003; Pelletier JG and Paré, 2004; Bauer et al, 2007; Dzirasa et al, 2011; Ghosh et al, 2013). This increase in synchronization is associated with freezing of fear -conditioned animals (Seidenbecher et al, 2003).

In addition to its central role in fear and anxiety, the amygdala also plays a role in associative learning with appetitive cues, aggression and sexual activity, as well as executive functions especially those associated with emotional stimuli (LeDoux, 2007).

1.4.3 Involvement in schizophrenia

Schizophrenia patients could exhibit abnormal emotional response including affective flattening, anxiety/depression in which the amygdala might play a role. Given the role of amygdala in aggression and sexual activity, it could also contribute to deficits of social interaction in

schizophrenia patients. No many studies on schizophrenia focused on the amygdala and findings on the amygdala seem controversial. Some early findings had suggested reduced GABAergic signaling in the BLA, but no recent work has focused on GABAergic system in the BLA (Benes, 2010). However, these controversial findings on amygdala might be due to diverse etiology of schizophrenia, whereas highly prevalent anxiety in schizophrenia patients and the correlation with premorbid anxiety and onset of psychosis suggest possible contribution of amygdala to the development of schizophrenia. The amygdala is extensively interconnected with hippocampus, PFC and other cortical regions, areas that show robust abnormalities in schizophrenia. Alterations could happen in connections between the amygdala and other brain regions and the amygdala could play a role in pathophysiology via these connections. Indeed, an animal model using pharmacological disinhibition of the amygdala shows decreased density of parvalbumin interneurons and decreased GAD65/67 expression in the hippocampus (Berretta et al, 2001, 2004; Gisabella et al, 2009).

1.5 PURPOSE OF STUDIES

Schizophrenia is a neurodevelopmental disorder arising from interaction of genetic predisposition and early life risk factors that lead to the onset of psychosis in late adolescence or early adulthood. Although stress is widely believed to contribute to the development of schizophrenia, patients do not necessarily experience more stressful life events preceding onset of psychosis. Instead, several studies on adolescents at high risk for schizophrenia have found a relationship of higher premorbid anxiety and impaired tolerance to stress with onset of psychosis.

Thus alleviating this hypersensitivity to stress might be an effective way to prevent the transition to psychosis.

Rats exposed during embryonic day 17 (E17) to the mitotoxin methyl azoxymethanol acetate (MAM) exhibit behavioral, pharmacological, and anatomical characteristics consistent with an animal model of schizophrenia. These rats exhibit increases in dopamine (DA) neuron population activity (i.e. the proportion of spontaneously active DA neurons) that correlates with the enhanced locomotor response to amphetamine, which is a behavioral correlate of psychosis. Furthermore, in agreement with findings from human at-risk subjects, these rats show impaired behavioral and endocrine responses to stress in juvenile and peripubertal period.

Stress is known to damage the hippocampus, a region commonly reported to be altered in postmortem and structural imaging studies of schizophrenia and which is proposed to underlie the DA system overdrive in the MAM model of schizophrenia. And as a central region to emotional regulation and stress responses, deficits in amygdala may leave susceptible individuals vulnerable to the deleterious effects of stress. Dysregulation of the hippocampus and amygdala may underlie impaired stress response in adolescent MAM rats, and the hypersensitivity to stress will in turn, exacerbate these deficits and contribute to development of symptoms related with schizophrenia. Therefore, we hypothesize that heightened response to stress contributes to the development of schizophrenia-like phenotypes in adult rats with MAM model of schizophrenia. We will investigate further if peripubertal MAM rats exhibit behaviors related with heightened response to stress and brain regions related with these behaviors. In addition, we will examine if treatment to alleviate this heightened response to stress during peripubertal period can prevent MAM rats from developing phenotypes related with schizophrenia later in life.

2.0 PERIPUBERTAL DIAZEPAM ADMINISTRATION PREVENTS THE EMERGENCE OF DOPAMINE SYSTEM HYPER-RESPONSIVITY IN THE MAM DEVELOPMENTAL DISRUPTION MODEL OF SCHIZOPHRENIA

2.1 INTRODUCTION

Schizophrenia is a neurodevelopmental disorder that afflicts approximately 1% of the population worldwide. Although there is a genetic linkage in the heritability of schizophrenia, it is clear that genetic predisposition alone is insufficient to account for the onset of this disorder. Instead, several investigators have proposed a two-hit model of schizophrenia, based on a genetic predisposition plus an early life risk factor(s) that lead to the onset of psychosis in late adolescence or early adulthood (Maynard et al, 2001). Several risk factors have been identified as related to schizophrenia; a major one that has shown a strong association with schizophrenia is stress (Walker and DiFiorio, 1997; Corcoran et al, 2003; Thompson et al, 2004). Thus, a number of retrospective studies reported that individuals with psychotic symptoms experienced more major stressful life events preceding the onset of psychosis (for review, see Phillips et al, 2007). Furthermore, stress sensitivity is likely associated with transition to psychosis. Owens et al (2005) reported that, in adolescents at high genetic risk for schizophrenia, those who showed a higher premorbid anxiety response to stress tended to be the same individuals who eventually converted to schizophrenia later in life. The impaired stress tolerance is associated with a range

of prodromal symtpoms and poor function over time (Devylder et al., 2013) and may be predictive of the development of psychosis (Yung et al, 2005).

Previous studies have shown that rats exposed during gestational day (GD) 17 to the mitotoxin methyl azoxymethanol acetate (MAM) exhibit behavioral, pharmacological, and anatomical characteristics consistent with an animal model of schizophrenia (Grace & Moore, 1998; Flagstad et al, 2004; Moore et al, 2006; for review, see Lodge and Grace, 2009). Moreover these rats exhibit increases in dopamine (DA) neuron population activity (i.e. the proportion of spontaneously active DA neurons) that correlates with the enhanced locomotor response to amphetamine (Lodge & Grace, 2007). In addition, consistent with the onset of psychosis in schizophrenia patients, the emergence of hyper-responsiveness to amphetamine develops after puberty.

Stress is known to damage the hippocampus (Mondelli et al, 2010, 2011), a region commonly reported to be altered in postmortem (FM, 1999) and structural imaging studies (Nelson MD, 1998) of schizophrenia and which is proposed to underlie the DA system overdrive in the MAM model of schizophrenia (Lodge and Grace, 2007). We have proposed previously that deficits in prefrontal cortical function could limit the ability of this structure to attenuate stress responses (Rosenkranz & Grace, 2001), leaving the susceptible individual vulnerable to the deleterious effects of stress (Thompson et al, 2004).

Given the evidence that stress and stress intolerance early in life may be a factor in the transition to schizophrenia in humans, we therefore hypothesize that attenuating the response to stress in the premorbid, peripubertal period may circumvent the process leading to a hyperdopaminergic state in the adult. We tested this hypothesis by administering an antianxiety

drug, diazepam across puberty and evaluated, in adult rats, the electrophysiological activity of dopamine neurons as well as the behavioral response to amphetamine.

2.2 MATERIALS AND METHODS

2.2.1 Animals

All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals by the USPHS and approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Pregnant Sprague-Dawley dams were obtained from Hilltop on gestational day 15 (GD15) and administered the mitotoxin methyl azoxymethanol acetate (MAM, 20mg/kg, i.p. obtained from Midwest Research Institute, Kansas City, MO) or saline on GD17. Litters were weaned on postnatal day 23 (P23) and housed in pairs. For the elevated plus maze test 3 litters with 3-6 pups from each litter were used for each experimental group (Sal- vs MAM-treated rats), and 2 litters with 2 pups from each litter were used for each experimental group (MAM:Veh vs MAM:DZ rats). For VTA dopamine neuron recordings, 5 litters with 1-3 pups from each litter were used for each experimental group. To assess locomotor responses to amphetamine, 6 litters with 1-4 pups from each litter were used for each experimental group.

2.2.2 Oral administration of diazepam

Diazepam (2mg tablets, obtained from Watson Laboratories, Inc., Corona, CA) was ground to power and mixed with sweetened condensed milk (Eagle Brand), sugar power and ground mini Nilla Wafers (Kraft Food). Approximately half of the pups from each litter were fed with this diazepam mixture across puberty, daily on 10 consecutive days (P31-40, 5mg/kg); others were fed the same mixture without diazepam. The oral administration route was chosen since it is less stressful than i.p. injections and better mimics the preferred route of drug administration to patients. In addition, it allows paired housing that is beneficial for rats with minimal separation (time necessary to consume the wafers, usually less than 15min) (Ferguson and Boctor, 2009). Male offspring were used for neurophysiology (P80-140) and locomotor tests (P62-77) as adults (Figure 1).

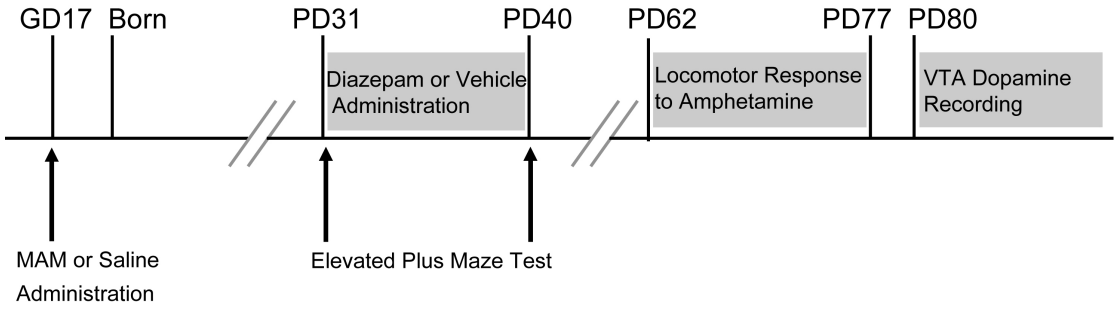

Figure 1. Experimental design.

2.2.3 In vivo recordings from VTA dopamine neurons

In vivo extracellular recordings were performed with investigators blinded to treatment. Rats were anesthetized with chloral hydrate and mounted on a stereotaxic frame (Kopf, Tujunga, CA). The body temperature was maintained at 37°C using a thermostatically controlled feedback heating pad (Fintronics, New Haven, CT). A burr hole was drilled in the skull overlying the right VTA. Extracellular recording microelectrodes were pulled from Omegadot 2.0 mm glass tubing on a Narishige P-5 vertical electrode puller, the tip broken back under microscopic control, and filled with 2M NaCl containing 2% Pontamine Sky Blue dye. The impedance of the electrodes in situ ranged from 6 to 15 MΩ. The stereotaxic coordinates for the VTA were 5.3mm posterior from bregma, 0.8mm lateral to the midline and 6.0 to 9.5mm ventral from the brain surface. Single-unit activity was filtered using a highpass filter at 30Hz and lowpass at 10kHz. All data analysis was performed using custom software (Neuroscope). Only neuronal activity with a signal-to-noise ratio greater than 3:1 and at least 3 min of stable spontaneous activity were used.

Six to nine vertical tracks, separated by 200 μm, were sampled in a predetermined pattern within the VTA of each rat. DA neurons were identified according to well-established electrophysiological features (Grace and Bunney, 1983; Ungless and Grace, 2012), which included the following criteria: 1) an action potential duration >2.2 ms; (2) slow firing rate (1-10Hz); and (3) irregular and burst firing patterns (the start of burst characterized by inter-spike interval <80ms, and the end of burst characterized by inter-spike interval >160ms). The activity of each identified DA neuron was recorded for at least 3 min. Three parameters of the population activity were analyzed: (1) the number of spontaneously active DA neurons per electrode track, (2) average firing rate and (3) the percentage of spikes that occurred in bursts (%SIB).

At the end of recordings, the recording site was marked via electrophoretic ejection of Potamine Sky Blue dye from the tip of the electrode (20 mA constant negative current, 30min). Rats were euthanized by an overdose of anesthetic; the brains were taken out, fixed for at least 48 hrs in 8% paraformaldehyde, cryoprotected in 25% sucrose, and sectioned for histological confirmation of the electrode sites.

2.2.4 Locomotor response to amphetamine

Adult rats were tested in an open-field chamber (Coulbourn Instruments, Allentown, PA) in which locomotor activity was determined by beam breaks and recorded with TruScan software (Coulbourn Instruments). All experiments were conducted at the same time of each day. Spontaneous activity was recorded for 30 minutes. After that, rats were injected with D-amphetamine sulfate (0.5mg/kg, i.p.) and their locomotor activity was recorded for another 90 min.

2.2.5 Elevated plus maze

Two groups of rats at PD31 and PD38-41 were run in the elevated plus maze . On PD38-41, diazepam (5mg/kg) was given to rats orally 90 minutes before the test. The apparatus had four elevated arms (50 cm above the floor), 50 cm long and 10 cm wide, arranged in a cross-like pattern, with two opposite arms enclosed by 40 cm high opaque walls and two open with a lip (1 mm thick and 5 mm high) and a central platform at their intersection (10 × 10 cm2) that permitted access to any of the four arms. Rats were handled for three consecutive days and habituated to the testing room one day before the test. Each rat was placed on the central

platform facing an open arm and the behavior was recorded for 5 min. The floor of the apparatus was cleaned between rats. The time spent in the open arms relative to that in the closed arms was used as an index of anxiety-like behavior.

2.2.6 Statistics and Analysis

Electrophysiological analysis of DA neuron activity was performed by custom-designed software (Neuroexplorer). Two-way ANOVA (MAM × diazepam) followed by Bonferroni post hoc test was used for comparison of DA neuron population activity. Locomotor activity was analyzed by TruScan software and compared using repeated measures three-way ANOVA (time as a within-subject factor, MAM and diazepam treatment as between-subject factors), followed by Bonferroni post hoc test. Activity of MAM and Saline rats on elevated plus maze was compared by Mann-Whitney rank sum test. All statistics were calculated using SigmaPlot (Systat Software, San Jose, CA) or SPSS statistics (IBM Corporation, Armonk, NY). All data are represented as the mean ±SEM.

2.3 RESULTS

2.3.1 MAM rats exhibited a higher anxiety level during adolescence that was reversed by diazepam treatment

The elevated plus maze test was performed on a group of rats at PD 31 prior to the administration of diazepam. Compared to rats with prenatal Saline treatment (n=12), MAM-

treated rats (n=14) spent significantly less time on open arms relative to closed arms (Figure 2A; $p<0.05$), indicating a higher level of anxiety. This is consistent with the higher level of anxiety reported for human adolescents at high risk for schizophrenia (Owens et al, 2005; Yung et al, 2005). Administration of diazepam acutely to MAM-treated rats at PD38-41, at a time point approximating the end of the diazepam treatment phase, effectively reversed the increased anxiety level in the elevated plus maze. Thus, MAM rats with diazepam administration 90 min before the test (MAM:DZ, n=4) spent significantly more time on open arms relative to closed arms (Figure 2B), and made significantly more open arm entries (0.75± 0.11) relative to closed arms compared to vehicle-treated MAM rats (MAM:Veh, n=4, 0.28± 0.08; MAM:Veh vs. MAM:DZ, $p<0.05$, t test).

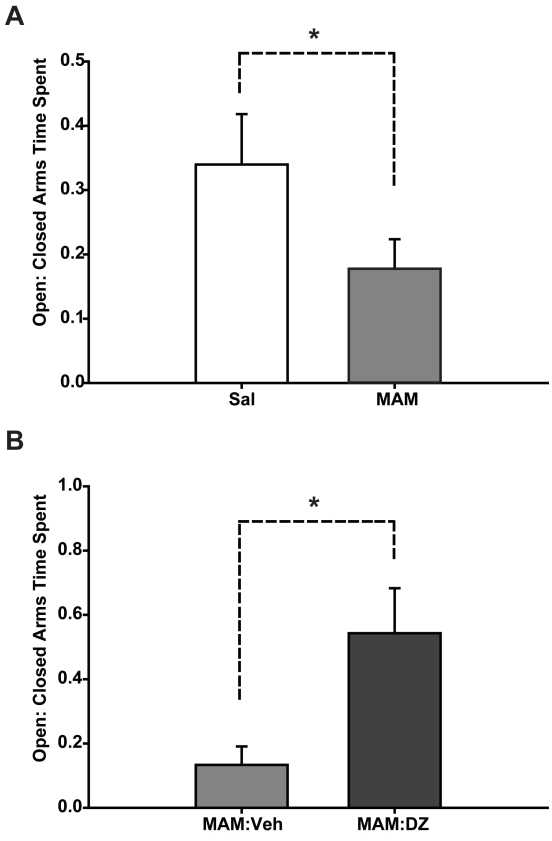

Figure 2. MAM rats exhibited a significantly higher level of anxiety compared to Saline rats during adolescence, which was reversed by diazepam administration.

2.3.2 Peripubertal diazepam administration prevented VTA dopamine hyperactivity in MAM-treated rats.

Consistent with what has been reported previously (Lodge and Grace, 2007; Gill and Grace, 2011), Sal:Veh (n=7 rats, 63 neurons) rats demonstrated an average of 1.1± 0.1 spontaneously active DA neurons per electrode track, an average firing rate of 3.3± 0.3 Hz and 20.9± 2.3% of spikes fired in bursts (Figure 3 A-C). Compared to Sal:Veh rats, recordings from MAM:Veh rats (n=7 rats, 120 neurons) showed a significantly greater number of spontaneously active DA neurons per electrode track (1.9± 0.1 cells/track, $p<0.001$), with no significant difference in average firing rate (3.4± 0.2 Hz) or percentage of spikes in bursts (23.0± 3.9%).

The number of spontaneously active DA neurons were significantly affected by prenatal MAM ($F_{1,24}=16.3$, $p<0.001$), peripubertal diazepam administration (5mg/kg, oral, PD31-40 daily; $F_{1,24}=7.2$, $p<0.05$), and their interaction ($F_{1,24}=13.4$, $p<0.01$; Two-way ANOVA MAM× diazepam). Compared to MAM:Veh rats, MAM:DZ rats (n=7 rats, 72 neurons) showed significantly fewer spontaneously active DA neurons (1.2± 0.04 cells/track; Bonferroni *post hoc* test). Furthermore, the numbers of DA neurons in the MAM:DZ vs. the Sal:Veh rats were not significantly different ($p>0.05$). In contrast, peripubertal diazepam treatment did not have a significant effect in Saline-pretreated animals. Sal:DZ rats (n=7 rats, 64 neurons) showed an average of 1.2± 0.1 cells per track, which was not significantly different from Sal:Veh rats ($t=0.7$, $p>0.05$). The firing rate (3.2± 0.2 Hz in MAM:DZ and 3.3± 0.3 Hz in Sal:DZ rats) and percentage of spikes in bursts (22.9± 3.7% in MAM:DZ and 20.0± 3.2% in Sal:DZ rats) did not differ significantly across all four groups ($p>0.05$, by two-way ANOVA).

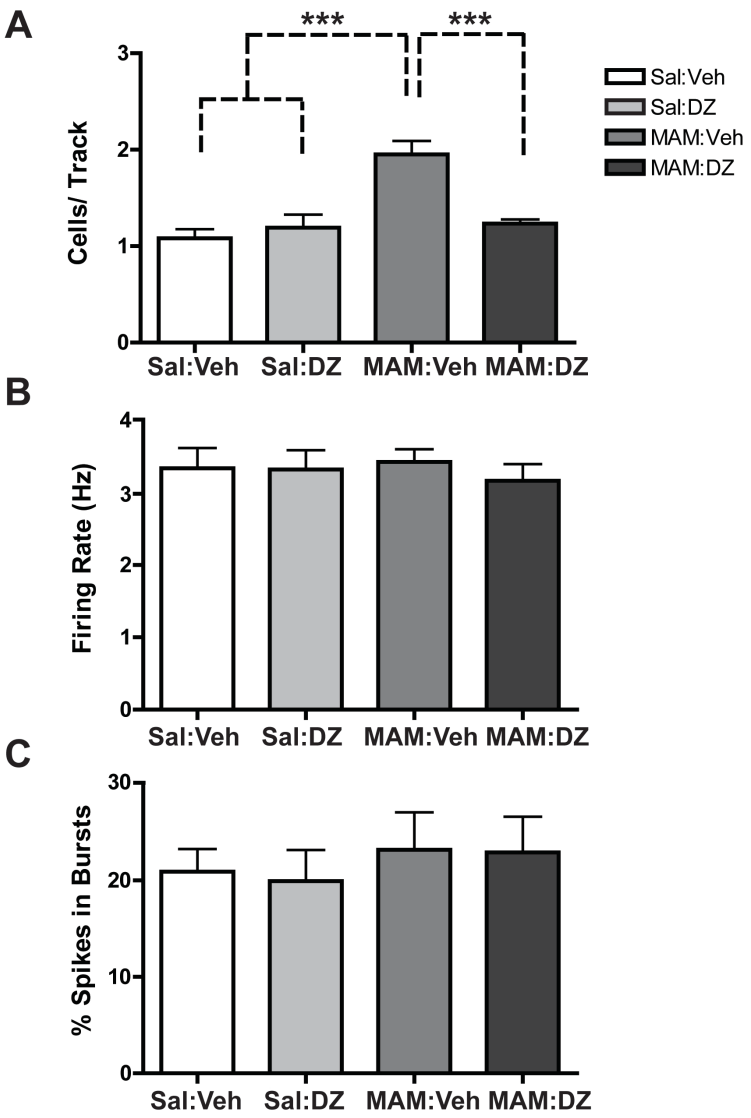

Figure 3. Peripubertal diazepam treatment prevented the pathological increase in the number of spontaneously active dopamine neurons in MAM-treated animals. ***, $p<0.001$

2.3.3 Peripubertal diazepam administration prevented the enhanced response to amphetamine in MAM-treated rats.

Previous studies have shown that rats treated with MAM on GD17 exhibited an enhanced locomotor response to amphetamine (Flagstad et al, 2004; Moore et al, 2006; Lodge and Grace,

2007). Consistent with those studies, MAM:Veh (n=10) rats showed significantly higher levels of locomotor activity in response to amphetamine administration (0.5 mg/kg i.p.) compared to controls (Figure 4A; $p<0.001$, Bonferroni post hoc test followed by repeated measures two-way ANOVA, $F_{1.36}=11.8$, $p<0.001$). Repeated measures of three-way ANOVA revealed a significant effect of time ($F_{8,288}=67.8$, $p<0.001$), MAM treatment ($F_{1,36}=22.0$, $p<0.001$), and the interaction of MAM and diazepam treatment ($F_{1,36}=10.6$, $p<0.01$). Locomotor activity was calculated within each bin (bin width=10 min). D-amphetamine injection is indicated by the dashed line. In contrast, MAM:DZ rats (n=10) showed a significantly lower level of amphetamine-stimulated locomotion compared to MAM:Veh rats ($p<0.05$), and were not significantly different from Sal:Veh rats ($p>0.05$). Furthermore, peripubertal diazepam treatment in Saline rats did not produce a significant alteration in amphetamine-stimulated locomotion (Sal:Veh vs Sal:DZ rats, n=9, $p>0.05$). The total movement distance after amphetamine administration revealed a similar result (Figure 4B), which was significantly affected by MAM ($F_{1,36}=21.5$, $p<0.001$) and the interaction of MAM and diazepam treatment ($F_{1,36}=10.9$, $p<0.01$). MAM:Veh rats showed a significantly higher total movement distance compared to MAM:DZ ($p<0.05$), Sal:Veh ($p<0.001$), and Sal:DZ rats ($p<0.01$). The spontaneous activity in a novel environment did not differ significantly among all four groups.

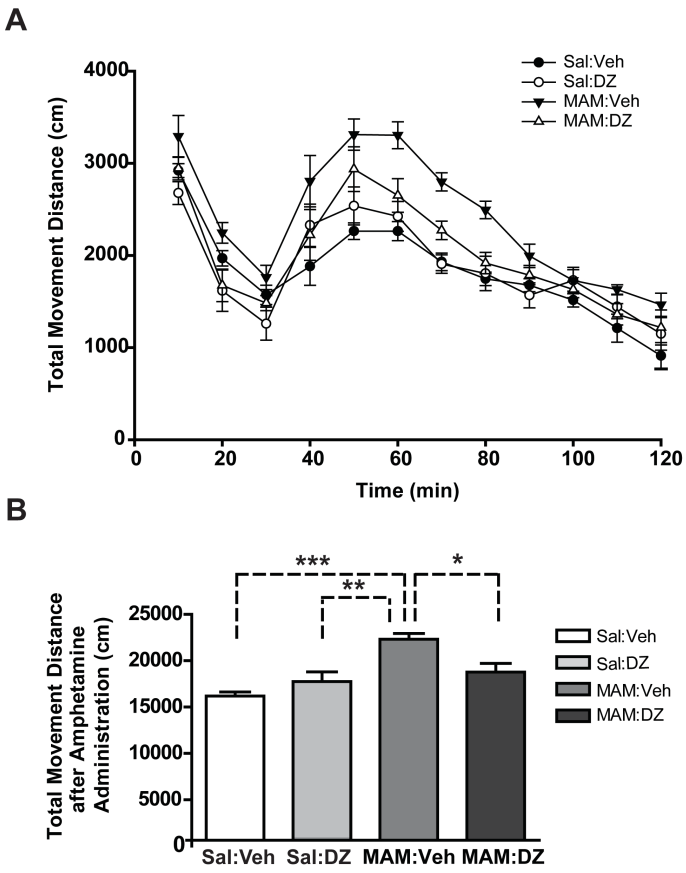

Figure 4. Rats treated peripubertally with diazepam showed an attenuation of the aberrant hyperlocomotor response to amphetamine that was observed in MAM-treated rats.

2.4 DISCUSSION

Although antipsychotic drugs have revolutionized the treatment of schizophrenia, in actuality this mode of treatment suffers from a low efficacy, the potential for producing untoward side effects, and the psychosis-induced alterations in brain function that may not be readily reversible pharmacologically (Lieberman et al, 2005). Moreover, studies have shown that the duration of untreated schizophrenia correlates with a worsened prognosis in patients (Hill et al, 2012). Therefore, a more effective approach to schizophrenia may be one of prevention. In this study,

we examined an approach that we propose may be an effective method to prevent the transition to psychosis in susceptible individuals.

2.4.1 Stress sensitivity in adolescence is associated with schizophrenia in humans

Stress is known to be a risk factor in schizophrenia (Walker and DiFiorio, 1997; Corcoran et al, 2003; Phillips et al, 2007) and is correlated with the propensity of at-risk individuals to transition to psychosis (Holtzman et al, 2012). Adolescents that convert to psychosis in later life showed higher sensitivity and intolerance to stress, a heightened anxiety level and a higher cortisol level (Owens et al, 2005; Yung et al, 2005; Walker et al, 2010; Corcoran et al, 2012; Devylder et al, 2013).

As for the biological response to stress, some studies (Walker et al, 2010) showed correlation between baseline cortisol levels in adolescents with schizotypal symptoms and severity of their schizotypal symptoms later in life. Enlargemeng of hypothalamus (Goldstein et al, 2007) and pituitary gland (Garner et al, 2005; Habets et al, 2012) were also found in schizophrenia patients and nonpsychotic relatives.

2.4.2 Stress sensitivity in MAM rats

Previous studies from our group showed that adult MAM rats are more vulnerable to stress exposure (Goto and Grace, 2006). In the current study, rats were not exposed to additional external stressors during the peripubertal period. Nonetheless, the fact that MAM rats showed high baseline anxiety levels peripibertally as assessed by the elevated plus maze suggests that even normal levels of stress (e.g. use of wire-bottom caging, cage cleaning, transport and

handling, etc.) may have been sufficient to result in increased measures of anxiety as assessed by the elevated plus maze test. Whether the anxiety was endogenous or was due to a decreased tolerance to low-level stressors, administration of diazepam reversed the increased anxiety level observed in the MAM rats. This heightened anxiety during adolescence is proposed to contribute to the dopaminergic hyperresponsivity that occurs in the adult.

2.4.3 Stress sensitivity as a potential target for early intervention

There is substantial evidence to suggest that much of the biological and social change underpinning the development of schizophrenia may already be active in the pre-psychotic or prodromal phase. Indeed, the pre-psychotic phase may be the most sensitive part of the 'critical period' for preventive efforts (Phillips et al, 2002). To circumvent the effects of stress, we chose to use a potent anti-anxiety agent, diazepam. When administered at adolescence, diazepam significantly decreased the elevated anxiety of adolescent MAM rats as assessed by the elevated plus maze. By relieving anxiety and stress during the pre-psychotic period, we propose that diazepam may prevent the conversion to psychosis. Thus, in MAM rats peripubertal administration of diazepam was found to prevent the pathological increase in dopaminergic activity that is proposed to underlie psychosis in schizophrenia (Laruelle and Abi-Dargham, 1999). In this manuscript we focused on the anti-anxiety effects of diazepam; however, given the known disruption of the GABA system in MAM rats (Lodge et al., 2009) and in schizophrenia (Zhang and Reynolds, 2002; Lewis et al, 2012), part of the actions may be a restoration of GABA balance that may underlie schizophrenia. Indeed, administration of a selective $GABA_A$ alpha 5 benzodiazepine-like drug was found to reverse the hyperdopaminergic state in adult MAM rats (Gill et al, 2011).

While the peripubertal administration of diazepam was found to be effective in circumventing the markers of hyperdopaminergic function in rodents, it is likely that other stress-relieving interventions will also be effective. Thus, administration of antipsychotic drugs to adolescent rats in the maternal immune activation model of schizophrenia has been shown to circumvent the emergence of several correlates of schizophrenia in adult rats (Pointkewitz et al, 2011, 2012). While it is unclear which of several potential mechanisms may be involved, it is well-known that antipsychotic drugs do attenuate the cortisol response in schizophrenia patients (Walker et al, 2008), which would be consistent with our studies of diazepam. In addition, antidepressants, which also have anxiolytic properties, have been reported to be beneficial to treatment of prodromal schizophrenia in adolescents (Cornblatt et al, 2007). We propose that controlling the effects of stress in at-risk individuals during the prodromal period may be an effective means to prevent the transition to schizophrenia later in life.

One issue that this raises is who should be given the treatment? Individuals that are at risk for schizophrenia can be identified based on genetic background and family history (Sullivan et al, 2003; Straub and Weinberger, 2006). Structured interviews for evaluating psychosis risk such as the Structured Interview for Psychosis Risk Syndromes (SIPS; Miller et al, 2003) and the Comprehensive Assessment of At-Risk Mental States (CAARMS; Yung et al, 2005) also contribute substantially toward creating a reliable and valid system for identifying risk prior to psychosis onset. Moreover, studies show that at-risk individuals that transition to psychosis often show increased stress responsivity in childhood and adolescence (Owens et al, 2005; Yung et al, 2005; Corcoran et al, 2012; Devylder et al, 2013). Given these data, we propose that individuals that are at risk for schizophrenia could be tested for their response to stress, and simply treat the stress hyper-reactivity. Both pharmacological and non-

pharmacological interventions that are effective at reducing stress, drawing from our data, may also be an effective means to prevent the eventual transition to psychosis later in life.

2.4.4 Mechanisms underlying the effects of stress on dopamine hyperactivity

Our previous studies showed that the increase in DA neuron activity was due to hyperactivity in the ventral hippocampus, since pharmacological inactivation of this structure (Lodge and Grace, 2007) or administration of a hippocampal-selective GABAA alpha 5 positive allosteric modulator (Gill et al, 2011) could reverse this increase in dopamine neuron population activity as well as the augmented increase in locomotor response to amphetamine. These data are also consistent with clinical results showing hyperactivity in the limbic hippocampus that correlated with psychosis (Malaspina et al, 1999; Medoff et al, 2001; Molina et al, 2003), an increase in amphetamine-induced dopamine release in schizophrenia that correlated with exacerbation of psychosis (Laruelle and Abi-Dargham, 1999), and in ultra-high risk individuals alterations in hippocampal glutamate levels that occurred in concert with increased presynaptic indices of dopaminergic function (i.e., fluorodopa uptake; Schobel et al, 2009; Stone et al, 2010; Howes et al, 2011). Taken together, these data are consistent with a model in which hippocampal hyperactivity leads to increases in dopamine neuron population activity, rendering the system hyper-responsive to stimuli (Lodge and Grace, 2007). The hippocampal hyperactivity is proposed to be due to a loss of parvalbumin interneurons in the MAM model (Penschuck et al., 2006, Lodge et al., 2009), which is consistent with postmortem observations in schizophrenia brains (Zhang and Reynolds, 2002).

We propose that the loss of parvalbumin interneurons occurs secondary to stress-induced damage of the hippocampus. Stress has been shown to lead to hippocampal atrophy (Magarinos

and McEwen, 1995; Lupien et al, 1998; Conrad et al, 1999). Moreover, studies have shown that pharmacological activation of the amygdala, a region known to be involved in stress and anxiety, will lead to decreases in hippocampal parvalbumin neuron number (Barretta et al, 2001).

Altogether, our findings indicate that treating the deleterious effects of stress, which may be magnified in at-risk individuals, during the peripubertal period may circumvent the cascade of events that leads to the emergence of psychosis in the adult.

3.0 ABNORMAL AMYGDALA ACTIVITY IN MAM MODEL OF SCHIZOPHRENIA: PERSISTENT NORMALIZATION FOLLOWING PERI-PUBERTAL DIAZEPAM ADMINISTRATION

3.1 INTRODUCTION

Schizophrenia is a debilitating mental disorder that affects 1-1.5% of the world population. Patients with schizophrenia show symptoms across several domains, including psychotic as well as cognitive and emotional symptoms. Underlying these behavioral symptoms are abnormalities across multiple brain regions, in which prefrontal cortex (Volk and Lewis, 2002) and hippocampus (Nelson et al., 1998; Benes, 1999) are the most strongly implicated. Another region, the amygdala, has garnered increasing attention particularly as it relates to the pathophysiology of schizophrenia.

Abnormal activities of amygdala have been reported in schizophrenia patients in response to emotional stimuli (Hall et al, 2008), as well as alterations in amygdala structure, molecular profile and physiological activity (Benes, 2010). Given its pivotal role in emotional function, alterations in the amygdala may underlie emotional and social deficits in schizophrenia. Moreover, the BLA is extensively interconnected with the hippocampus and PFC (Sah et al, 2003), with malfunction in these circuits leading to cognitive deficits related to stress and emotion (Kim and Diamond, 2002). An animal model of schizophrenia was generated by

pharmacologically disinhibiting the amygdala, which leads to a decreased density of hippocampal parvalbumin and GAD65/67 expression (Berretta et al, 2001, 2004), similar to that observed in schizophrenia patients (Volk and Lewis, 2002).

Schizophrenia is widely believed to be a neurodevelopmental disorder, in which alterations start early in development, until the emergence of psychosis usually during late adolescence and early adulthood. Both genetic and environmental factors contribute to the development of schizophrenia. Among those risk factors, stress/sensitivity to stress are thought to contribute to the transition into psychosis. Aberrant cortisol level, stress intolerance and heightened anxiety level were observed in subjects at risk for schizophrenia and are correlated with onset of psychosis later in life (Owens et al, 2005; Yung et al, 2005; Walker et al, 2010; Corcoran et al, 2012; Devylder et al, 2013). Similarly, in rats with the MAM-E17 developmental disruption model of schizophrenia, we observed heightened anxiety-like behaviors and higher sensitivity to stress (a blunted yet not adaptive corticosterone level, more freezing and more ultrasonic vocalizations in response to footshock exposure) in juvenile and adolescent MAM rats (Du and Grace, 2013; Zimmerman et al, 2013). The neurodevelopmental model of MAM-E17 was introduced by our group (Moore et al, 1998) and has been adopted by a number of other research groups. By administering a mitotoxin methyl azoxymethanol acetate (MAM) to pregnant dams on gestational day 17, these MAM-E17 offsprings exhibit behavioral pharmacological, and anatomical characteristics consistent with an animal model of schizophrenia (for review, see Lodge and Grace, 2009).

The amygdala plays a pivotal role in emotional regulation, stress and anxiety (LeDoux, 2007). Changes in amygdalar function may underlie the increased sensitivity to stress and contribute to the development of schizophrenia. Increased activity of the amygdala is accompanied with increased cue conditioning and anxiety-like behaviors (LeDoux, 2007). Synchronization in the amygdala is also related with these emotional processes (Sneidenbechet et al, 2003; Pelletier JG and Paré, 2004; Likhtik et al, 2014).

The amygdala is composed of several heterogenous nuclei. The basolateral complex (BLA) consists of lateral (LA) and basolateral (BA) nuclei. This is the primary region that connects with the prefrontal cortex (PFC) and hippocampus (Sah et al, 2003). In this study, we examined both single unit and synchronizing activities of amygdala in control and MAM-treated rats. The hyperactivity of BLA was already present in peri-pubertal rats and persisted to adulthood. Correlated with heightened activity of the BLA, MAM rats showed more anxiety and cue conditioning. The administration of anti-anxiety drug diazepam during peri-pubertal period has been shown to prevent the hyperdopaminergic state in adult MAM rats (Du and Grace, 2013). In this study, we showed that this peri-pubertal diazepam administration also leads to a persistentreduction in anxiety-like behavior and normalization of hyperactivity of BLA in adult MAM rats.

3.2 MATERIALS AND METHODS

3.2.1 Animals

All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals by the USPHS and approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Pregnant Sprague-Dawley dams were obtained from Harlan on Gestational Day (GD) 15 and administered with MAM (20mg/kg, i.p., Midwest Research Institute, Kansas City, MO) or saline on GD 17. Litters were weaned on postnatal day 23 (P23) and housed two or three per cage. Only male offspring were used in this study. Animals were housed in a normal light cycle (lights on at 7am and off at 7pm) unless otherwise specified.

3.2.2 Oral administration of diazepam

Diazepam (2mg tablets, Watson Laboratories, Inc., Corona, CA) was ground to powder and mixed with sweetened condensed milk (Eagle Brand), sucrose powder and ground mini Nilla Wafers (Kraft Food). For BLA single unit recordings from peripubertal rats, rats were fed with an acute dose of diazepam (5mg/kg) mixture or mixture without diazepam 90 min before behavioral tests or recordings. For tests on adult rats, diazepam (5mg/kg) or vehicle was administered during the peri-pubertal period with once daily administration on 10 consecutive days (PD31-40). Approximately half of the pups from each litter were fed with this diazepam mixture, others were fed with the same mixture without diazepam. Rats remained drug free after this 10 day administration, and were used for recordings or behavioral tests after they reach adulthood (i.e., after PD70). The oral administration route was chosen because it is less stressful

than i.p. injection especially for rats at the peri-pubertal age who are more sensitive to stress, as well as better mimicking the preferred route of drug administration to patients (Ferguson and Doctor, 2009).

3.2.3 Acute surgeries and in vivo extracellular recording from basolateral amygdala of anesthetized rats

Rats were anesthetized with chloral hydrate (400mg/kg, i.p.) and supplemented periodically to maintain suppression of the hind limb withdrawal reflex. The body temperature was maintained at 37 ºC using a thermostatically controlled feedback heating pad (Fintronics, New Haven, CT). A burr hole was drilled in the skull overlying the right BLA. Extracellular recording micro electrodes were pulled from omega dot 2.0 mm glass tubing on a Narishige P-5 vertical electrode puller, the tip broken back under microscopic control, and filled with 2M NaCl containing 2% Chicago Sky Blue dye. The impedance of the electrodes in situ ranged from 7 to 15 MΩ. The stereotaxic coordinates for BLA were 3.3 mm posterior to bregma and 5.0 mm right from the midline suture for adults, and 2.5 mm posterior and 4.5 mm right for peri-pubertal rats (Sherwood and Timiras, 1970; Paxinos and Watson, 1986). Single-unit activity was collected using a high pass filter at 30 Hz and low pass at 10 kHz. Spontaneously active cells (firing rate higher than 0.1 Hz) were searched 6.0 to 8.5 mm ventral from brain surface. Once identified, activity was recorded for at least 3 minutes by Labchart software (ADInstrument, Colorado Springs, CO).

3.2.4 Elevated plus maze

The elevated plus maze is positioned 50 cm above the floor. It has four arms, each 50 cm long and 10 cm wide, arranged in a cross-like pattern, with two opposite arms enclosed by 40 cm high opaque walls, the other two open without any walls, and a central platform at their intersection (10 X 10 cm2). Rats were handled for 3 days and habituated to the testing room the day before the test. During the test, each rat was placed on the central platform facing an open arm, and its movement was recorded for 5 minutes. The floor of the maze was cleaned thoroughly between rats. The percentage of time spent in the open arms and percentage of entries into the open arms were calculated as indices of anxiety-like behaviors.

3.2.5 Survival surgeries

Rats for LFP recordings were housed in reverse light cycle (7:00 PM light on, 7:00 AM light off). Survival surgical procedures were performed in a semisterile environment. Adult (250-350 g) and peri-pubertal rats (PD 28-31, 120-150 g) were anesthetized with isoflurane (5% in oxygen for induction, around 2% for maintenance) and placed in a stereotaxic apparatus using blunt ear bars. Body temperature was maintained at 37 ºC . Custom length (40 mm) polyimide-insulated stainless steel wire electrodes (Plastics one: E363/3, 0.15 mm) were implanted bilaterally into BLA (posterior 2.9 mm, left or right 5.0 mm, ventral 8.6 mm from bregma for adults; posterior 2.5 mm, left or right 4.5 mm, ventral 8.3 mm for peri-pubertal rats) (Sherwood and Timiras, 1970; Paxinos and Watson, 1986). A screw attached to teflon insulated stainless steel wire (Plastics One, E363/20, 0.56 mm) was fixed above lambda as ground. Another 4 to 6 (for adults), or 2 (for peri-pubertal rats) anchor screws were attached to the skull. Electrodes and connectors

(E363, Plastics One) were secured with dental cement to the anchor screws. Rats were housed individually after surgery, and received carprofen (5mg/kg, i.p.) and tylenol (1 ml in 10 g chow) for 3 days. Behavioral trainings started 7 days after surgery.

3.2.6 Standard fear conditioning and local field potential (LFP) recordings from the BLA

Rats that were used for fear conditioning and local field potential (LFP) recordings were housed in a reverse light cycle (light on 7:00 PM and off 7:00 AM). The behavioral testing was performed during the active period (between 7:00 AM and 7:00 PM). Rats were trained for a standard auditory fear conditioning paradigm. A glass open chamber (Coulbourn Instruments) was used for recording in which rats were habituated to the chamber for 2 days (Day 1 and 2). On Day 3, rats were exposed to 10 trials (intertrial interval ranging from 45 to 100 s) of 2 s tone presentation that ends with a 1 s footshock (0.45 mA) in a different operant chamber (MedAssociates). Twenty four hours after conditioning (Day 4), rat were placed in the same glass open chamber as they were in Day 1 and 2. LFP electrodes were connected with a 12 channel commutator (Plastics One) to a 4 channel amplifier (A-M system, model 1700). After at least 20 minutes habituation to the glass chamber, rats were exposed to 10 trials of 2 s tones alone and LFP signals in BLA were recorded. LFP signals were amplified 10 k times, filtered (low pass: 1 kHz and high pass: 1Hz) and digitized at 1000 Hz in LabChart (ADInstrument). Freezing behaviors were defined as immobility that last at least 2 s, and hand scored for 45 s after tone presentation.

3.2.7 Histology

For BLA single unit recording from anesthetized rats, at the end of recordings, recording sites were marked via electrophoretic ejection of Chicago Sky Blue dye from the tip of the electrode. Rats were then overdosed with chloral hydrate and decapitated. For LFP recordings from awake, freely moving rats, after recordings, recording sites were marked by passing a 0.1 mA current for 10 s through the electrode and rats were euthanized with carbon dioxide. Brains were removed and fixed in 8% paraformaldehyde in PBS for 72 hrs, immersed in 25% sucrose in PBS, sliced and stained with Cresyl Violet. Recording sites were verified by microscopic localization of the blue dye mark and electrode track. Only cells located in the basolateral amygdala complex (both basolateral amygdala and lateral amygdala) were used for analysis.

3.2.8 Data analysis and statistics

For BLA single unit activity, only cells that exhibited action potential durations longer than 1ms and firing rates lower than 1Hz were included in order to preferentially record from projection neurons. There are indeed some projection neurons that fire over 1Hz; however using these criteria, the population included for analysis was found in other studies to be comprised of almost exclusively putative projection neurons (Rainnie et al, 1993; Rosenkranz and Grace, 1999; Likhtik et al, 2006). Firing rates were transformed via a logarithmic function to fit a normal distribution. One-way ANOVA with Bonferroni post hoc test was performed to compare Sal- and MAM-treated peri-pubertal rats; for adult rats, a two-way ANOVA was used to compare effects of prenatal MAM and peri-pubertal diazepam administration.

Analysis of local field potential activity was performed using the chronux package (www.chronus.org) in MATLAB (MathWorks). In brief, LFP oscillatory activity was filtered (3–20 Hz), detrended and Fourier transformed into different frequency bands. Continuous multitaper time-frequency spectral analyses were conducted for data from 2 s before to 4 s after onset of the tone. LFP signals were normalized to those 2s before the onset of the tone. Theta oscillations were average from 4 to 8 Hz. The ratio of theta power after the onset of the tone versus baseline was calculated by normalizing theta power during the 2 s tone presentations to that 2 s before the onset of the tone. Trials with saturated signals were excluded and the remainders within each animal were averaged. Animals with data from less than 5 trials were excluded from analysis. We did not see a significant difference between left and right BLA; we thus pooled data from left and right BLA. For rats with data from bilateral BLA, we only used those recorded from right side.

All statistical tests were performed by SigmaPlot (SigmaStat Software), Matlab (MathWorks), or SPSS (IBM Software). Data are presented as mean± SEM.

3.3 RESULTS

3.3.1 Increased level of anxiety in adult MAM treated rats as tested in the elevated plus maze

In our previous work, MAM-treated rats showed a higher level of anxiety as tested in the elevated plus maze at the peri-pubertal age (Du and Grace, 2013). In this study, we conducted the elevated plus maze test on adult rats and observed a similar increased in the level of anxiety,

as indicated by a decrease in the percentage of time spent in the open arms and the percentage of entries into open arms. MAM rats with peripubertal diazepam treatment spent a significantly higher percentage of time in open arms and made significantly more entries into open arms compared to MAM:Veh rats, which were not significantly different from Sal- treated rats (Figure 5). For percentage of time spent in open arms, two-way ANOVA revealed a significant effect of MAM ($F_{1,42}$=8.006, $p<0.01$), diazepam ($F_{1,42}$=4.264, $p<0.05$) and their interaction ($F_{1,42}$=5.806, $p<0.05$). The percentage of entries into open arms: Two-way ANOVA revealed a significant effect of MAM ($F_{1,42}$=4.600, $p<0.0105$), and the interaction between MAM and diazepam ($F_{1,42}$=4.413, $p<0.05$). Bonferroni tests followed by this two-way ANOVA revealed a significant difference between MAM:Veh (n=10) and MAM:DZ (n=17) within MAM groups, and a significant difference between MAM:Veh and Sal:Veh (n=10) within Veh groups but not between Sal:Veh and Sal:DZ (n=9), or Sal:DZ and MAM:DZ.

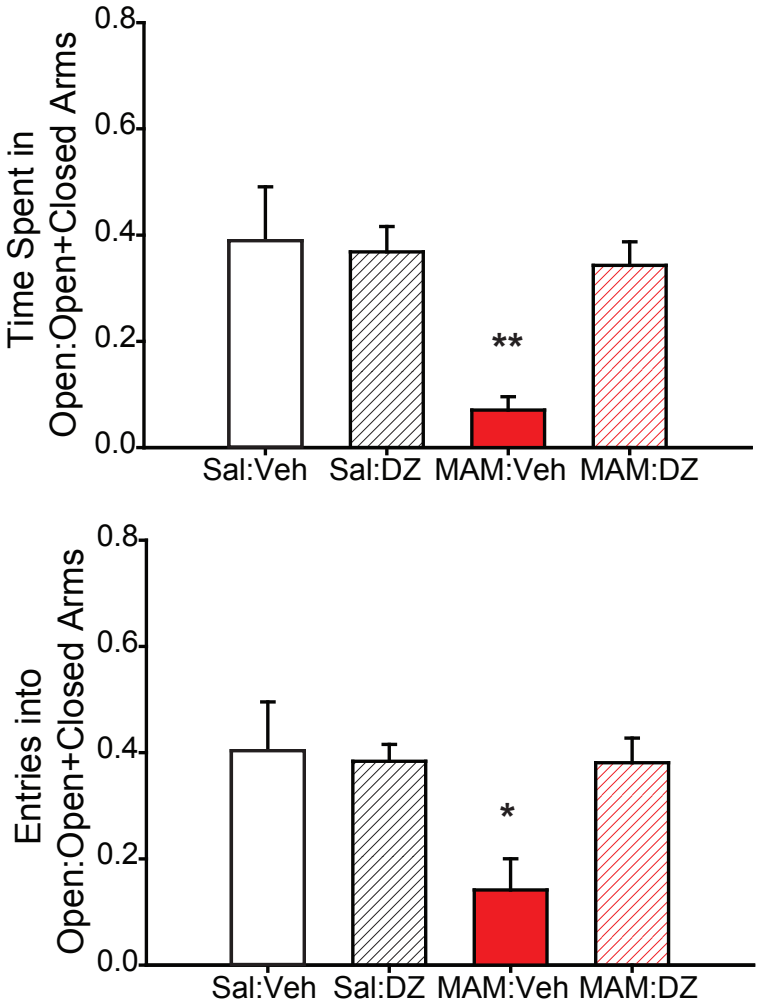

Figure 5. Peripubertal diazepam administration decreased the heightened level of anxiety in adult MAM rats.

3.3.2 Increased firing rate of BLA neurons in both peripubertal and adult MAM treated rats

The basolateral amygdala (BLA) is a key component in the fear/anxiety circuit (LeDoux, 2007). Therefore, alterations in anxiety level of MAM treated rats should be reflected by alterations in

BLA activity. The heightened level of anxiety was observed in MAM rats at both peripubertal and adult ages. We thus conducted in vivo single unit recordings from the BLA of anesthetized rats during both the peripubertal period and adulthood.

Similar to that reported previously (Rosenkranz and Grace, 1999), firing rates of spontaneously active BLA neurons are low (n=13, 0.11±0.02 Hz in peripubertal control rats, Figure 6; n=33, 0.21±0.03 Hz in adult control rats, Figure 7). At the peripubertal age of PD35-42, MAM rats exhibited a significantly higher spontaneously firing rate (n=30, 0.41±0.04 Hz) compared to Sal rats. We also tested the effect of a single dose of diazepam on BLA firing rate in peripubertal rats and found that in MAM rats an acute administration of diazepam (5 mg/kg, oral) caused a significant inhibition of firing rate (n=12, 0.14±0.02 Hz) compared to Sal rats. One-way ANOVA among all three groups revealed a significant effect of treatment ($F_{2,53}$=28.425, $p<0.001$), and following Bonferroni t-tests revealed significant difference between MAM and Sal rats (t=9.471, $p<0.001$), and between MAM and MAM with acute diazepam administration (t=4.914, $p<0.001$) and between Sal and MAM with acute diazepam administration (t=2.642, p=0.028).

In adult rats, two-way ANOVA among all four groups revealed a significant effect of both MAM ($F_{1,123}$=19.778, $p<0.001$) and diazepam treatment ($F_{1,123}$=7.741, $p<0.01$). BLA neurons in MAM:Veh rats exhibited a significantly higher spontaneously firing rate (n=39, 0.41±0.04 Hz) compared to Sal:Veh (t=4.096 $p<0.001$), and MAM:DZ (n=29, 0.28±0.04 Hz, t=2.732, p=0.043). No significant difference was found between other groups.

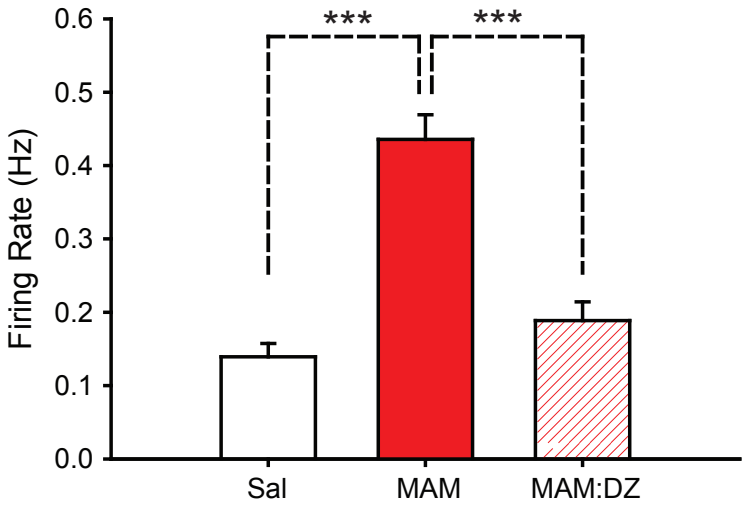

Figure 6. MAM rats exhibited heightened spontaneous activity of BLA neurons peri-pubertally (PD35-42), which was reduced by acute administration of diazepam.

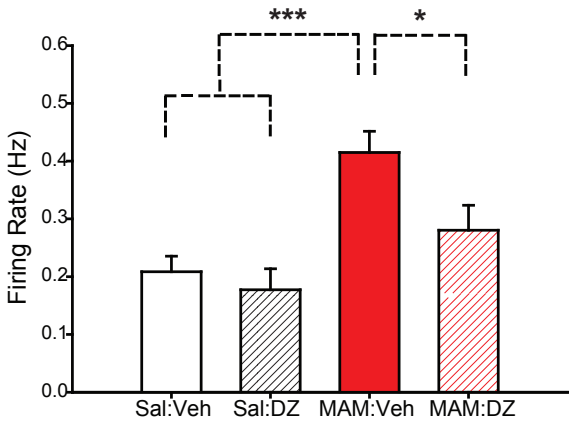

Figure 7. Peripubertal administration of diazepam decreased heightened BLA spontaneous activity in adult MAM rats.

3.3.3 Increased theta power during conditioned tone presentation in adult MAM rats and reduction of this increase following peri-pubertal diazepam administration.

Oscillatory activities reflect signal processing activity and correlate with behavioral responses. The oscillatory activity in BLA is related to fear and anxiety (Sneidenbechet et al, 2003; Pelletier JG and Paré, 2004; Likhtik et al, 2014). Therefore, in addition to recording single unit activity, we also recorded local field potentials (LFPs) from the BLA of awake, freely moving rats. LFP recordings were conducted after rats were trained in a standard acoustic fear conditioning paradigm. Since they were recorded in a different context from where they were conditioned, the response we observed should reflect only cue-induced but not contextual fear conditioning. We did not observe significant effects of MAM on freezing behavior 45 s following onset of the conditioned tones (Figure 8B), which is consistent with lack of difference in locomotor activity in previous studies (Lodge et al., 2009). However, peripubertal administration of diazepam significantly reduced freezing behaviors (n=6-10, $F_{1,26}$=5.45, p=0.028).

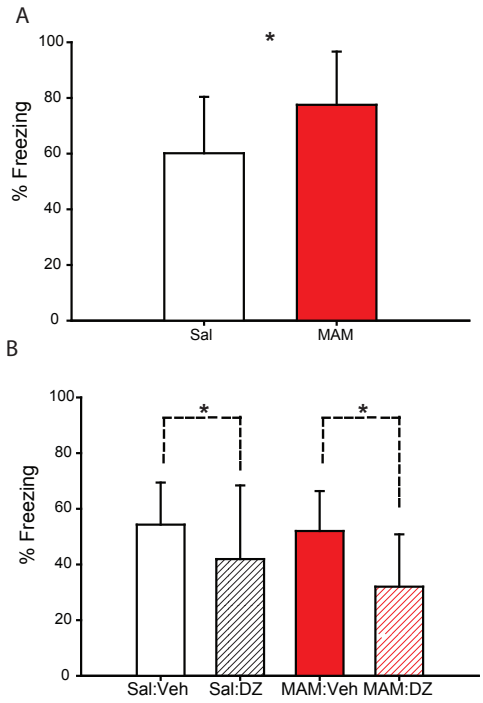

Figure 8. Peripubertal (A) but not adult (B) MAM-treated rats exhibited significantly more freezing to the conditioned tone compared to Sal-treated ones.

LFP signals recorded when these rats were exposed to conditioned tones (conditioned stimuli, CS, Figure 9) revealed an increase in theta power during the 2 s period of tone presentation compared to baseline (2 s before the tone presentation), which is similar to what has been reported previously (Sneidenbechet et al, 2003; Likhtik et al., 2014). Examples of raw traces in one trial from one adult Sal and one adult MAM rats were shown in Figure 9A. The bar represents tone onset from 0 to 2 s. LFP signals were Fourier transformed to different frequency bands and normalized to baseline (2 s immediately before the onset of the tone). The power spectrograms from example Sal and MAM animals are represented as z score in heat maps of Figure 9B. The average power spectrograms of theta frequency (4-8 Hz) from Sal- and MAM-treated rats are shown in Figure 9C, in which red line represents MAM rats, while blue

represents Sal rats. This increase in theta power during tone presentation is correlated with freezing behaviors following the tone in Sal-treated rats (R^2=0.75, Figure 9C); however, no such correlation was observed in MAM-treated rats. Comparing all four groups of adult rats, no differences in the peak theta power was observed among these animals but the increase in theta power averaged across 2-s tone presentation (Sal:Veh: n=6, 141%± 10%; Sal:DZ: n=6, 165% ± 18%; MAM:Veh: n=5, 225% ± 31%; MAM:DZ: n=5,158% ± 7%, Figure 9E) showed significant effects of MAM ($F_{1,\ 18}$= 4.42, p=0.05) and the interaction of MAM and diazepam treatment ($F_{1,18}$=6.03, p=0.024) by two-way ANOVA. Benforroni post hoc test revealed a significant effect of diazepam within MAM group (MAM:Veh vs MAM:DZ, t=2.243, p=0.025) and a significant effect of MAM within vehicle group (MAM:Veh vs Sal:Veh, t=3.222, p=0.005) but no significant effect on other groups.

However, peripubertal rats exhibited differences in behavior compared to adults (Figure 8A). Peripubertal MAM rats (PD38-42) exhibited significantly more freezing behaviors (78% ±19%, n=14) compared to Sal rats (60% ± 20%, n=12, t_{24} = -2.254, p=0.034). The increase in theta power during tone presentation in Sal rats are not significantly different from that in adult rats, but the increase in MAM rats was significantly less than that of adults (peripubertal Sal: 124% ± 28%, n=7; MAM: 99% ± 14%, n=7) as revealed by two-way ANOVA comparing peripubertal or adult, Sal or MAM treatment (significant effect of age ($F_{1,21}$=2.12, p<0.001) and the interaction of age and MAM treatment ($F_{1,21}$=13.6, p=0.001) and post hoc tests (peripubertal vs adult within MAM, t=5.865, p<0.001). And peripubertal MAM- and Sal- treated rats not significantly differ on theta power increase during tone presentation.

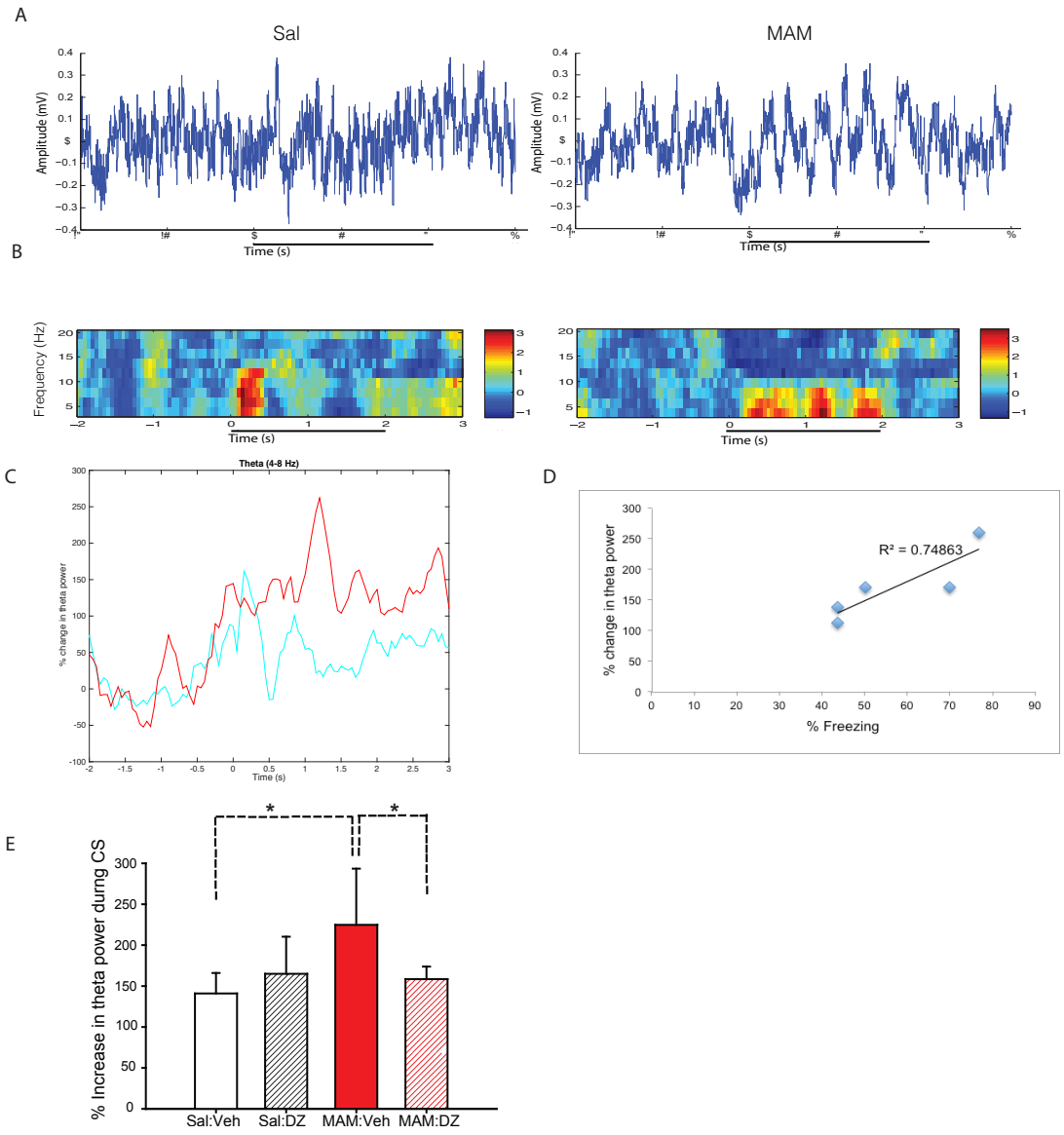

Figure 9. Adult MAM-treated rats exhibited a larger increase in CS induced theta power than Sal rats and MAM rats with peripubertal diazepam treatment.

3.4 DISCUSSION

In current study, we found a significantly higher level of anxiety in adult MAM rats as tested in the elevated plus maze, similar to that observed in rats tested peripubertally (Du and Grace,

2013). Anxiety is also known to be prevalent in schizophrenia patients, with a comorbidity rate of 38.3% between anxiety disorders and schizophrenia based on a meta-analysis, and even higher co-occurrence of anxiety symptoms in schizophrenia (Achim et al., 2011). This rate varies in different studies, but schizophrenia is a highly heterogeneous disorder itself. The heightened level of anxiety observed in our MAM model of schizophrenia supports this comorbidity of anxiety and schizophrenia.

Altered function in the amygdala has been advanced as an underlying condition in both anxiety and schizophrenia (LeDoux, 2007, Benes, 2010). Increased anxiety and fear conditioning are related to hyperactivity in the BLA. This includes both single unit activity as well as LFP recordings (Sneidenbechet et al, 2003; Pelletier JG and Paré, 2004; Likhtik et al, 2014). In this study, neurons in the basolateral amygdala (BLA) of MAM rats at both peripubertal and adult ages exhibited a significantly higher spontaneously firing rate. In addition, adult MAM rats showed a significantly larger increase in theta power in response to a conditioned tone as tested in a standard fear conditioning paradigm. The relationship between increase in BLA theta power and freezing behaviors in response to conditioned tone was confirmed by correlation observed in Sal-treated rats (Fig 9D). We did observe a significantly large increase in theta power in adult MAM rats; however, this increase was not reflected in freezing behaviors of these rats. The discrepancy in theta power increase and freezing behaviors between Sal and MAM rats could reflect the contribution of other brain regions to learning or expression of fear in these rats. Indeed, both ventral hippocampus and the PFC exhibited abnormal function in MAM-treated rats (Goto and Grace, 2006; Lodge and Grace, 2007; Lodge et al., 2009) and they both are important structures involved in fear learning (Rosenkranz and Grace, 2002; Maren and Quick, 2004).

Interestingly, the difference in BLA activity in MAM rats started as early as in the peripubertal period and persisted to adulthood, which is consistent with the heightened level of anxiety observed in both peripubertal (Du and Grace, 2013) and adult rats. Indeed, although the onset of psychosis usually occurs during late adolescence and early adulthood, alterations in behaviors and brain structures initiated significantly earlier. Subjects at risk for schizophrenia demonstrate a variety of prodromal symptoms including alterations in emotional and cognitive functions (Johnstone et al, 2005), as well as hypermetabolism in the hippocampus (Schobel et al., 2013). Similarly, in our MAM-treated rats, the hyperlocomotion in response to psychostimulants is only observed in adult but not younger rats (Moore et al., 2006). However, at juvenile and peripubertal ages, these MAM rats have already exhibited a significant degree of pathology: they are more sensitive to stress and more anxious (Du and Grace, 2013; Zimmerman et al, 2013), andthe reduction of parvalbumin expression in the hippocampus is already present in juvenile MAM rats (Chen et al, 2014; Gill and Grace, 2014).

Our observation that only peripubertal MAM-treated rats exhibited significantly more freezing behaviors than Sal-treated ones might suggest a higher sensitivity to footshock stress, which is supported by previous findings showing a higher level of footshock-induced vocalization and freezing and a blunted corticosterone response that failed to accommodate with repeated footshock that was observed only in juvenile and peripubertal rats (Zimmerman et al., 2013). Indeed, the pre-psychotic phase may be the most sensitive part of the 'critical period' at which intervention could be effective at preventing the transition to psychosis later in life (Phillips et al, 2002). Based on the widely accepted two-hit hypothesis for the development of schizophrenia, a genetic background could cause the individual to be more vulnerable to the impact of stressful life events, thereby precipitating the subsequent onset of psychosis. However,

schizophrenia subjects may not necessarily experience more stressful life events but instead experience these events as more uncontrollable and intolerable (Thompson et al, 2004;van Winkel et al, 2008). Subjects at risk for schizophrenia exhibited higher level of anxiety and stress intolerance (Owens et al, 2005; Yung et al, 2005), which is correlated with onset of psychosis later in life. The early-emerged increase in anxiety in MAM rats mimics what was observed in these at-risk subjects. The present study together with others (Du and Grace, 2013; Zimmerman et al, 2013; Gill and Grace, 2014) enables the MAM model to be used to develop strategies for early treatment and prevention of schizophrenia. An interesting possibility arises when considering the impact of diazepam in preventing MAM-associated emergence of the putative psychosis state. Unlike genetic disorders such as Huntington's disease, in which the gene causes the disorder, in schizophrenia genetic risk factors instead only predispose an individual to stress. In the MAM rat, this predisposition appears to occur via an increase in the deleterious effects of stress. It may be that genetic risk in schizophrenia patients may also reflect an increased vulnerability to environmental risk factors, rather than causing the disease per se.

In previous work, we confirmed the anti-anxiety effect of diazepam in peripubertal MAM rats, and that this anti-anxiety effect peripubertally prevented the emergence of hyperactivitiy of VTA dopamine neurons and the heightened locomotor response to amphetamine challenge in adult MAM-treated rats (Du and Grace, 2013). In the present study, we continued to examine the effect of this peripubertal (PD31-40) administration of diazepam, focusing on anxiety-like behaviors and related BLA activities. We observed a persistent anti-anxiety effect of this administration in adult MAM rats, after remaining drug free for at least a month. Thus, those with previous diazepam treatment during the peripubertal period exhibited a significantly lower level of anxiety, lower spontaneously firing rate and less increase in conditioned tone-induced

theta power compared to those who did not receive such treatment. Although we did not have control experiments in which diazepam was administered to adult rats to confirm the unique actions of diazepam given during the peripubertal period in rats, we do have previous studies supporting heightened stress sensitivity in juvenile and peripubertal rats (Zimmerman et al., 2013). This peripubertal period, corresponding to mid- to late- adolescence in humans, is a critical period for prefrontal cortical and inhibitory circuit development, both involved in the pathophysiology of schizophrenia (Volk and Lewis, 2002). The heightened response to stress in peripubertal MAM rats would make these rats more vulnerable to the debilitating effect of stress and influence normal development of these structures. For example, parvalbumin interneurons are sensitive to stress (Czeh et al., 2005; Hu et al., 2010) and would thus be impaired. And indeed, the loss of parvalbumin interneurons in the hippocampus was observed in juvenile rats and this loss progressed to adulthood (Gill and Grace, 2014). Loss of parvalbumin in the hippocampus is related to anxiety (Quarta et al, 2005). Therefore, the long lasting effect of peripubertal diazepam administration to reduce anxiety and hyperactivity of the BLA, and also to prevent the emergence of the hyperdopaminergic state, could occur via protecting parvalbumin interneurons from further stress-induced damage. Genetic factors or prenatal risk factors predispose individuals to schizophrenia, e.g. leading to a hypersensitivity to stress as observed in at-risk subjects, in which dysfunction of the amygdala may be one of the mechanisms underlying these risks. This hypersensitivity to stress in turn exacerbates deficits, and contributes to development of schizophrenia. However, with early treatment to alleviate this heightened response to stress, like peripubertal administration of diazepam, individuals will be less vulnerable to the effect of stress, and thus less likely to have further impairment led by stress and to eventually transit into psychosis.

4.0 LOSS OF PARVALBUMIN INTERNEURONS IN THE VENTRAL SUBICULUM OF THE HIPPOCAMPUS OF RATS WITH MAM MODEL OF SCHIZOPHRENIA IS ATTENUATED BY PERIPUBERTAL ADMINISTRATION OF DIAZEPAM

4.1 INTRODUCTION

Loss of parvalbumin (PV) positive interneurons in PFC and the hippocampus is one of the most robust findings from postmortem brains of schizophrenia patients (Volk and Lewis, 2002; Zhang et al, 2002). Interneurons that express parvalbumin are fast spiking interneurons and control gamma oscillations (Sohal et al 2005). In accordance with this loss of PV interneurons, deficits in gamma oscillations were also observed in schizophrenia patients (Spencer et al, 2004, 2008; Trautner et al, 2006). The loss of PV interneurons has been reported in several animal models of schizophrenia, including neonatal hippocampal lesion (Cabungcal et al., 2014), maternal immune activation (Piontkewitz et al., 2012), amygdala disinhibition (Berretta et al., 2009) and MAM-E17 models (Penschuck et al, 2006; Lodge et al, 2009; Chen et al, 2014; Gill and Grace, 2014).

Rats exposed during embryonic day 17 (E17) to a mitotoxin, methyl azoxymethanol acetate (MAM) exhibit behavioral, pharmacological, and anatomical characteristics consistent with an animal model of schizophrenia (for review, see Lodge and Grace, 2009). These rats exhibit increases in dopamine (DA) neuron population activity (i.e. the proportion of DA neurons firing spontaneously), which is thought to underlie the behavioral correlates of psychosis

as well as the enhanced locomotor response to amphetamine and other psychostimulants, (Flagstad et al., 2005, Moore et al., 2006). In addition, consistent with the onset of psychosis in schizophrenia patients, the emergence of hyper-responsiveness to amphetamine develops after puberty. Heightened population activity of VTA dopamine neurons is driven by a pathologically excessive activity of hippocampus (Lodge & Grace, 2007), which could be due to decreased density of parvalbumin-positive interneurons in the hippocampus (Lodge et al., 2009). In addition, the loss of parvalbumin interneurons in the hippocampus of MAM treated rats begins in juvenile (PD25) rats, and persists into adulthood (Gill and Grace, 2014; Chen et al, 2014).

In previous work, we have observed a hyperactivity of the basolateral amygdala (Lister et al.) in both peripubertal and adult rats in the MAM model of schizophrenia. The BLA also contains a high density of parvalbumin interneurons, innervating projection neurons and regulating the firing of projection neurons (McDonald and Betette, 2001; Muller et al, 2006; Wolff et al, 2014). The BLA hyperactivity we observed previously might be due to disinhibition resulted from reduction of GABAergic signaling. Therefore, we examined if there was a change in parvalbumin interneurons in BLA of MAM treated rat.

Peripubertal administration of diazepam (PD31-40) has been shown to prevent the emergence of hyperdopaminergic state in adult MAM- treated rats by alleviating the heightened level of anxiety in MAM rats during the peripubertal period (Du and Grace, 2013). However, the cellular mechanism by which this protection occurs is unclear. Parvalbumin interneurons, especially those in the hippocampus, are sensitive to chronic stress (Czeh et al, 2005, 2015; Hu et al, 2010). Therefore, one possibility is that this treatment to alleviate hyperresponsivity to stress could protect parvalbumin interneurons from further loss. In this study, we will investigate if

peripubertal administration of diazepam can restore the level of parvalbumin interneurons in MAM treated rats.

4.2 MATERIALS AND METHODS

4.2.1 Animals

All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals by the USPHS and approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Pregnant Sprague-Dawley dams were obtained from Harlan on Gestational Day (GD) 15 and administered MAM (20mg/kg, i.p., Midwest Research Institute, Kansas City, MO) or saline on GD 17. Litters were weaned on postnatal day 23 (P23) and housed two or three per cage. Only male offspring were used in this study. Animals were housed in a normal light cycle (lights on at 7am and off at 7pm) unless otherwise specified.

4.2.2 Oral administration of diazepam

Diazepam (2mg tablets, Watson Laboratories, Inc., Corona, CA) was ground to powder and mixed with sweetened condensed milk (Eagle Brand), sucrose powder and ground mini Nilla Wafers (Kraft Food). For BLA single unit recordings in peripubertal rats, rats were fed with an acute dose of diazepam (5mg/kg) mixture or mixture without diazepam 90 min before surgeries and recordings. For tests on adult rats, diazepam (5mg/kg) or vehicle was administered during the peri-pubertal period with one daily administration on each of 10 consecutive days (PD31-40).

Approximately half of the pups with MAM administration from each litter were fed with this diazepam mixture; others were fed with the same mixture without diazepam. Rats remained drug free after this 10-day administration. The oral administration route was chosen because it is less stressful than i.p. injections especially for rats at the peri-pubertal age that are more sensitive to stress, as well as better mimicking the preferred route of drug administration to patients (Ferguson and Doctor, 2009).

4.2.3 Tissue preparation

Adult rats (PD83) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed, fixed in 4 % paraformaldehyde for half an hour, and stored in 0.1 M phosphate buffer. Before slicing, sections were cryoprotected in 25% sucrose in 0.1 M phosphate buffer for 48 h, and sliced into 60 μm coronal sections. Every fourth section from the basolateral amygdala and the ventral subibulum of the hippocampus was used for immunohistochemistry, with the starting section randomly picked. Adjacent sections were used for Cresyl violet staining.

4.2.4 Immunohistochemistry

Free floating sections were rinsed in 0.01M PBS, and treated with 0.5% sodium borohydride for 30 min, and then 1% hydrogen peroxide to block endogenous peroxidase activity. Sections were blocked for 1 hr in 3 % normal goat serum, 0.5% Triton in 0.01 M PBS and then incubated with primary antibody (rabbit polyclonal anti-PV, 1:5000, PV27, Swant) in the same blocking solution overnight at room temperature. Sections were then incubated in biotinylated secondary

antibody (goat anti-rabbit, 1:500, Vector) for 1 hr and then in VECTASTAIN Standard ABC Kit for 40 min. Sections were rinsed in TBS, and the immunoperoxidase reaction was performed in 3'3'-diaminobenzidene (DAB) solution containing 0.1% hydrogen peroxide for 5 min. Sections were mounted on gelatin coated glass slides, and after dehydration through 50%, 75%, 95%, 100% alcohol and xylene, coverslipped with permount.

4.2.5 Stereology

Parvalbumin positive cells were counted in the right basolateral amygdala (Lister et al.) and ventral subiculum of the hippocampus (vSub) using an unbiased stereological method: optical fractionator (West et al, 1991). The optical fractionator provides unbiased estimates of neuron numbers, which are free of assumptions about neuron size and shape.

The BLA comprises the lateral nucleus, the basolateral nucleus, and the basomedial nucleus (Sah et al, 2003). Boundaries of the BLA were defined according to an atlas of the rat brain (Paxinos and Watson, 1986) and previous studies (Chareyron et al., 2012). For the anterior part of BLA, the upper and medial border was determined by the different cytoarchitecture of the BLA which contrasted with the caudate-putamen and central nucleus of the amygdala; for the posterior part, the BLA is adjacent to lateral ventricle. Laterally it is bordered by the external capsule.

For vSub, only the pyramidal cell layers, where most PV interneurons were located, were counted. The division between vSub and CA1 subregion of the hippocampus was defined by an abrupt widening of the pyramidal layer, where the superficial cells of the pyramidal cell layer are no longer observed. And the border between the vSub and presubiculum cortex is defined by the sharp transition to the smaller cells of the presubiculum (West et al, 1991).

Sections were examined using a Nikon microscope installed with a motorized stage (Ludl Electronic Products, Hawthorne, New York), a digital camera (QImage), using StereoInvestigator 9 software (MicroBrightfield, Inc., Williston, Vermont). Researchers were blind to treatment of animals. Boundaries of regions of interest were traced on cresyl violet stained sections, modified on peroxidase-stained sections under 2.5X lens, and further refined under 10X lens. Counting was performed using a 60X objective. For the BLA, a 200 mm * 200 mm counting frame was used in a grid size of 325 mm * 325 mm. For vSub, a 150 mm * 150 mm counting frame was used in a grid size of 300 mm * 300 mm. The average section thickness was 19.9 mm, therefore a 12 mm height was chosen for counting, with 3 mm for the top guard zone and the rest as bottom guard zone.

4.2.6 Data analysis and statistics

Estimates of neuron population and volume were determined by optical fractionator probes of the software, and density was calculated as population divided by volume. For the BLA, population, volume, and density were compared between Sal- and MAM-treated rats via the Student t-test. For the vSub, comparisons were made among three groups (Sal, MAM, MAM with peripubertal diazepam administration) using one-way ANOVA followed by Bonferroni post hoc tests. All statistical tests were performed using SigmaPlot (SigmaStat Software), or SPSS (IBM Software). Data are represented as mean ± SEM.SEM.

4.3 RESULTS

4.3.1 MAM treated rats did not exhibit loss of parvalbumin interneurons in the basolateral amygdala (BLA)

A total of 11 to 12 sections were counted for the BLA of each rat?. The Coefficient of Error Gunderson (m=0) ranges from 0.07 to 0.12. Shrinkage of sections was 64% ± 1%, rendering an average thickness of sections after shrinkage of 20.2 ± 0.6 μm. Both small, medium to large size parvalbumin positive (PV) interneurons were observed in BLA and vSub, with roundor fusiform shape, which is consistent with previous findings (Pitkänen et al, 1995; Kemppainen and Pitkänen, 2000) (Figure 10). For population estimates, the Coeffient of Error Gunderson (m=0) ranged from 0.07 to 0.12. The number of parvalbumin positive (PV) interneurons averaged 3283 ± 175 in Sal-treated rats (n=5), which was to that reported previously (Rostkowski et al., 2009). We did not observe significant differences between Sal- and MAM-treated rats in PV neuron number in BLA (MAM rats, 3797 ± 372, n=5, t8=-1.247, p>0.05, Student t-test). In addition, no significant differences between Sal- and MAM- treated rats in volume or density of PV interneuron in the BLA was observed.

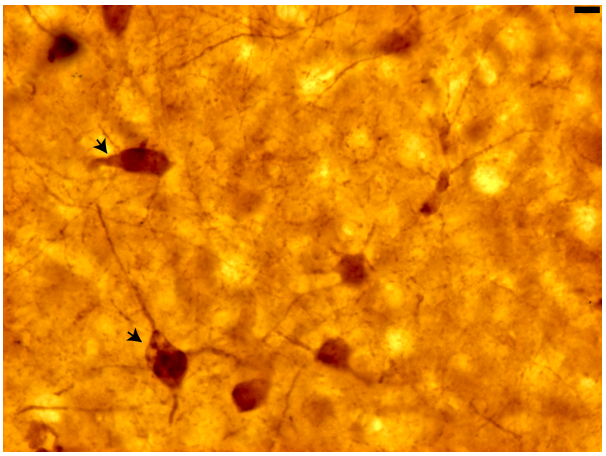

Figure 10. Examples of parvalbumin positive (PV) interneurons in focus under 60X lens. Arrows points to PV cells in focus; scale bar represents 10 μm.

4.3.2 Loss of parvalbumin interneurons in ventral subiculum of the hippocampus (vSub) of MAM- treated rats was attenuated by peripubertal administration of diazepam.

A total of 9 to 11 sections for Sal-treated rats and 7 to 9 for MAM-treated rats (with or without peripubertal administration of diazepam) were counted throughout the ventral subiculum of the hippocampus (vSub). The Coefficient of Error Gunderson (m=0) ranged from 0.07 to 0.16. The average section thickness was 19.6 ± 0.05 mm after accounting for a shrinkage of 67% ± 1%, and was not significantly different among Sal-, MAM- treated rats, and MAM rats with peripubertal administration of diazepam (MAM: DZ rats). An average of 5072 ± 319 parvalbumin positive (PV) interneurons were counted in each of the Sal-treated rats (n=4), consistent with previous studies (11610 for both ventral and dorsal subiculum, Lister et al, 2011). However, in MAM- treated rats, the number of PV interneurons counted was significantly less (n=5, 2348 ± 158, one-way ANOVA, $F_{2,10}$=36.349, p<0.001, following Bonferroni test, t=8.376, p<0.001). MAM rats with peripubertal administration of diazepam had significantly more PV interneuron compared to MAM rats without such treatment (n=4, 3094 ± 227, t=2.293, p=0.045); however, this number was still significantly lower compared to Sal- treated rats (t=5.771, p<0.001, Figure 11A). A similar pattern was observed for the density of PV interneurons, in which MAM rats with peripubertal administration of diazepam had significantly higher density of PV interneurons but still significantly lower than that of Sal- treated rats (Figure 11B, Sal: 4805.7± 363.8 cells /mm^3, MAM: 2380.8± 171.8 cells /mm^3; MAM: DZ: 3222.3± 276.9 cells

/mm^3; $F_{2,10}$=21.338, p<0.001, Sal vs MAM, t=6.494, p<0.001; MAM: DZ vs MAM, t=2.254, p=0.048; MAM:DZ vs Sal, t=4.023, p=0.002). No significant difference in volume of the vSub was noted among these three groups.

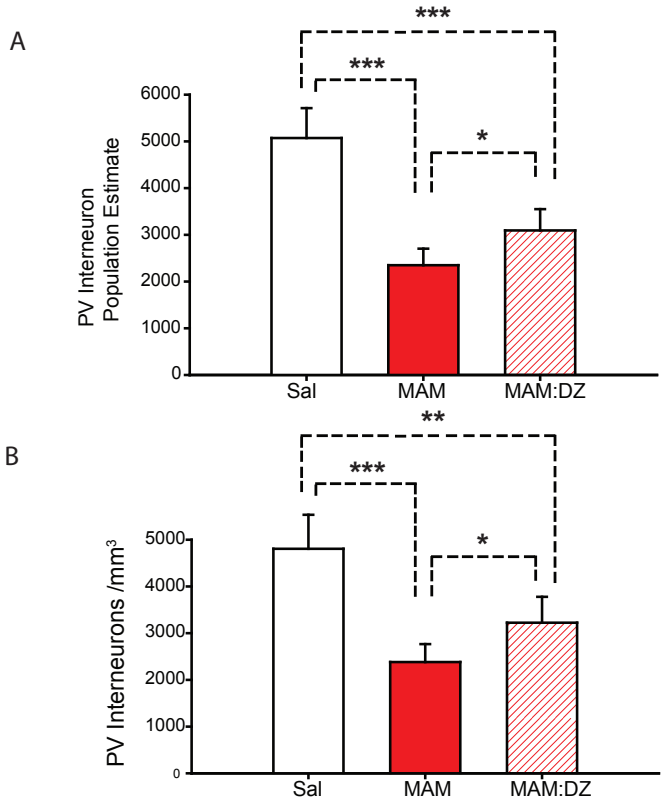

Figure 11. Peripubertal diazepam administration attenuated loss of parvalbumin interneurons in MAM rats.

4.4 DISCUSSION

In this study, by using an unbiased stereological method, we observed a loss of parvalbumin interneurons in the hippocampus of rats with MAM model of schizophrenia, consistent with

previous findings (Penschuck et al, 2006; Lodge et al, 2009; Chen et al, 2014; Gill and Grace, 2014). The reduction in parvalbumin protein expression in the hippocampus begins as early as juvenile (PD25) and shows diverse patterns in different subregions of the hippocampus. Although increasing from juvenile to adulthood, the level of parvalbumin protein expression in the ventral hippocampus (subregions not differentiated) of MAM rats remained approximately 50% lower than that of Sal-treated controls, as detected by Western blot (Chen et al, 2014). Studies using immunohistochemistry on adult MAM treated rats reported a 30% loss of parvalbumin neurons (Penschuck et al., 2006) without differentiating between ventral and dorsal hippocampus. The different levels of reduction reported could be due to different detection methods (i.e., labeled cells vs protein) or the difference between dorsal and ventral hippocampus. Indeed, MAM-E17 treatment exerts a bigger impact on PV interneurons in the ventral hippocampus, in which a reduced level of PV expression is paralleled with a reduced number of PV cells, in contrast to a reduced PV expression but not loss of PV cells in the dorsal hippocampus (Gill and Grace, 2014). Moreover, in this study by Gill and Grace (2014), subregions of the hippocampus were identified, with only the dentate gyrus and CA3 subregions of the hippocampus used and the level of reduction in the ventral dentate gyrus and CA3 reported to be about 20%. The loss of PV interneurons in the vSub appears to be larger as reported by another study, in which a 50% reduction of PV cell density in the ventral but not dorsal subiculum of the hippocampus was observed (Lodge et al., 2009). In the present study, a 50% reduced density of PV cells was also observed in the vSub. It appears likely that the impact of MAM-E17 is different among subregions of the hippocampus, with the vSub being among the most highly affected subregions.

One caveat of this study is lack of difference observed in the volume of the vSub between Sal- and MAM- treated rats. Reductions of about 5% in hippocampal volume have been reported in both postmortem and imaging studies of schizophrenia brains (Bogerts 1997; Heckers and Konradi 2002). Similarly, rats treated with MAM are reported to have a slightly but significantly smaller hippocampus (Moore et al., 2006). However, we did not observe significant differences in the volume of the vSub between Sal- and MAM- treated rats. Several factors could contribute to this discrepancy. First, the sample size is quite small and underpowered to detect the small change in the hippocampal volume (power=0.217). Nonetheless we did notice a reduction in volume in the anterior-posterior dimension, as vSub from MAM treated rats contained fewer longitudinal sections (7 to 9 for MAM vs 9 to 11 for Sal). In addition, although the borders of vSub were easily recognized in cresyl violet stained sections, for the hippocampus of MAM treated rats pyramidal cell layers are more diffuse and disorganized (Moore et al, 2006; Penschuck et al, 2006; Matricon et al, 2010). A more diffuse pyramidal cell layer could cause one to overestimate the size of the counting areas in MAM-treated rats compared to Sal- treated ones. Because we counted only pyramidal cell layers where most PV interneurons are located, the increased area in the pyramidal cell layer alone could mask an overall reduction in the volume of the whole vSub. Moreoever, a more diffuse and disorganized pyramidal cell layer across the hippocampus makes the division between CA1 and vSub difficult to demarcate. Thus we cannot rule out whether part of CA1 regions may have been included as part of the vSub in the MAM treated rats. These factors could all contribute to the lack of significant differences observed in the volume of the vSub between Sal- and MAM- treated rats.

In contrast to the obvious reduction of PV interneuron population and density in the vSub of MAM rats, we did not observe any significant difference in either number or density of PV

interneurons in the BLA, or in BLA volumes of these rats. The specific impact of MAM-E17 on the hippocampus could be due to a different time course of BLA and hippocampal development. The peak of cell proliferation in the hippocampus is around E16 to E18 (Bayer, 1980), but BLA is actively developing between E14 and E16 (Berdel et al, 1997). As a mitotoxin, the effect of MAM in disrupting cell division lasts 12 to 24 hr after injection, when the hippocampus is actively developing but the BLA has already undergone development. In addition, whether the volume of the amygdala decreases in schizophrenia patients is still controversial, in that many studies reported a reduction of temporal lobe structures (hippocampus and amygdala) but the volume of the amygdala alone does not seem to be reduced (Chance et al, 2002). Moreover, whether parvalbumin interneurons in the BLA of schizophrenia patients are altered is still unclear. Some early findings had suggested reduced GABAergic signaling in the BLA, but no recent work has focused on GABAergic system in the BLA (Benes, 2010). Reduction in PV expression in the BLA has been observed in some animal models of schizophrenia (MK-801, Romón et al, 2011; PDGFR-β KO, Nakamura et al, 2015) but not others (Pollard et al., 2012).

Parvalbumin interneurons, especially those in the hippocampus, are sensitive to stress. Hippocampal PV neurons were reduced in both social defeat and chronic mild stress models (Czeh et al, 2005, 2015; Hu et al, 2010). Interestingly, this reduction is different among subregions of the hippocampus (e.g. ventral CA1 is more sensitive compared to other subregions of the ventral hippocampus in these depression models, Czeh et al, 2015). A hyperresponsivity to stress was observed in MAM rats during the peripubertal period, e.g. enhanced baseline level of anxiety and lack of accommodation of the corticosterone response to chronic stress (Du and Grace, 2013; Zimmerman et al, 2013). This hyperresponsivity to stress would cause these rats to more likely perceive everyday life events as stressful, which could damage PV interneurons in

the hippocampus secondary to amygdala activation. This could also explain the incomplete reversal of the reduced PV interneuron numbers in MAM rats with peripubertal administration of diazepam. By alleviating the hyperresponsivity to stress, this treatment prevents further loss of PV interneurons. However, density of PV interneurons is already lower in MAM rats at PD25, whereas diazepam is administered later on PD31-40. In addition, the volume of the hippocampus is smaller (Moore et al., 2006). A reduction in density together with reduction in hippocampal volume results in an even large decrease in PV interneuron population, which cannot be fully restored by peripubertal administration of diazepam on PD31-40. Nonetheless, perpubertal administration of diazepam was effective at protecting some PV interneurons from loss. By doing so, this treatment was able to keep the system under a threshold so the cascade that eventually leads to emergence of hyperdopaminergic state in MAM- treated rats will not be triggered. Perhaps an earlier and longer developmental time point for diazepam administration would be more efficacious in preventing the pathology.

5.0 GENERAL DISCUSSION

5.1 SUMMARY OF FINDINGS

There are two major aims of these studies. The first one is to investigate alterations in response to stress of rats with MAM model of schizophrenia during peripubertal period and to explore brain regions related with altered response to stress. In these peripubertal MAM rats, we have observed heightened level of anxiety as tested in elevated plus maze, increased cue induced freezing in standard fear condoning paradigm, and increased firing rate of basolateral amygdala (BLA) neurons. In addition, heightened anxiety-like behavior and hyperactivity of BLA persists into adulthood.

The second aim is to investigate whether treatment to alleviate anxiety in peripubertal period (i.e. anti-anxiety drug diazepam administered on PD31-40) could prevent phenotypes related with schizophrenia in adult MAM rats. MAM rats with treatment of diazepam during peripubertal period for 10 days on PD31-40 did exhibit a normalized VTA dopamine population activity, an attenuated amphetamine-induced, and less reduction of parvalbumin interneuron in the ventral subiculum of the hippocampus. In addition, this peripubertal administration of diazepam has a persistent effect to reduce anxiety-like behaviors in adult MAM-treated rats, as well as to reduce increased firing in BLA neurons and increased theta oscillation induced by conditioned cues.

5.2 MAM MODEL TO STUDY DEVELOPMENTAL TRAJECTORY OF SCHIZOPHRENIA

In the past decade, a lot of efforts have been made to study the prodromal phase of schizophrenia, toward the goal of early treatment or even prevention for schizophrenia. Individuals that are at risk for schizophrenia can be identified based on genetic background and family history (Sullivan et al, 2003; Straub and Weinberger, 2006). Structured interviews for evaluating psychosis risk such as the Structured Interview for Psychosis Risk Syndromes (Miller et al, 2003) and the Comprehensive Assessment of At-Risk Mental States (Yung et al, 2005) also contribute substantially toward creating a reliable and valid system for identifying risk before psychosis onset.

As a neurodevelopment model of schizophrenia, MAM-E17 model has its unique advantage for studies on developmental trajectory and early interventions for schizophrenia. Our studies have utilized this model to investigate symptoms in MAM rats before adulthood, and to test if early intervention could prevent emergence of schizophrenia-like symptoms. Indeed, many other researchers have also focused on progressive changes in phenotypes related with schizophrenia. Psychostimulants -induced hyper locomotion is present only in adult rats (Flagstad et al, 2004; Moore et al, 2006; Chen et al, 2014), which corresponds to the onset of psychosis often during late adolescence and early adulthood. One study reported that hyperactivity of the VTA dopamine neuron population happens before adulthood (Chen et al, 2014). Hyperactivity of the VTA dopamine neuron population is driven by hyperactivity of the hippocampus (Lodge and Grace, 2007). Although hippocampal hyperactivity in MAM rats at this age has not been examiner yet, loss of parvalbumin in the hippocampus has begun on PD25 (Chen et al, 2014; Gill and Grace, 2014). The cellular mechanism of hippocampal hyperactivity

is still unclear, but disinhibition resulting from loss of parvalbumin probably contributes to this hyperactivity (Lodge et al., 2009). In addition, abnormal cerebral blood volume increases and hypermetabolism observed in the hippocampus of at-risk human subjects, indicative of a hyperactivity of hippocampus that emerges early before onset of psychosis (Schobel et al, 2009; Schobel et al, 2013). However, the discrepancy in peripubertal MAM rats between hyperactivity of VTA dopamine neuron population and unaltered behavioral response to amphetamine requires further elucidation.

In another study, significantly reduced volume and neural density in PFC, as well as decreased expression of Gad1 mRNA were observed on adult rats (P60 or later) but not earlier (Mackowiak et al, 2013). Indeed, the pre-psychotic or prodromal phase may be the most sensitive period for schizophrenia (Phillips et al, 2002) and inhibitory circuitry in the PFC is under development during this peripubertal period (Volk and Lewis, 2002). Based on our findings of heightened anxiety in peribpuertal MAM rats and that anti-anxiety drug diazepam administered during peripubertal period prevent hyperdopmianergic state in adult MAM rats, heightened stress response may contribute to impairment in PFC of MAM rats.

5.3 ANXIETY AND ABNORMAL STRESS RESPONSE IN MAM MODEL OF SCHIZOPHRENIA

The Anxiety symptoms and disorders are highly prevalent throughout the course of schizophrenia with 38.3% comorbidity (Buckley et al., 2009, Achim et al., 2011). In addition, some evidence suggests that schizophrenia patients with comorbid anxiety disorders showed a decline in their subjective quality of life (Braga et a., 2005). Therefore, anxiety in schizophrenia

patients is an extra burden for their functioning and quality of life, which should be considered in their treatment. In addition, in more than 50% of schizophrenia patients, anxiety disorder preceded the onset of psychosis (Pokos and Castle, 2006). Indeed, studies on subjects at genetic risk of schizophrenia have found that adolescents who showed increased anxiety are those who developed into psychosis later in life (Jonhstone et al, 2005; Owens et al, 2005). Another group identified subjects at ultra high risk of schizophrenia using Comprehensive Assessment of At-Risk Mental States and found intolerance to stress in these subjects predictive of onset of psychosis (Yung et al, 2005). Correlation of impaired tolerance to normal stress and heightened anxiety in these subjects was also observed (Corcoran et al, 2012; Devylder et al, 2013).

Anxiety is a behavioral response to stress. We have observed heightened anxiety as measured in elevated plus maze, as well as increased fear conditioning in MAM rats. Abnormal response to stress in MAM rats was also reported in other studies. A blunted response in plasma corticosterone level in response to acute footshock, much less robust as seen in control rats, but lack of accommodation to chronic stress was observed in peripubertal MAM rats (Zimmerman et al, 2013). However, a substantial corticosterone response is necessary for homeostatic adaptation to stressors (McEwen and Gianaros, 2010; Rao et al, 2012). This persistent increase in corticosterone level will harm brain regions such as the hippocampus, which is highly sensitive to the level of corticosterone. In addition, MAM rats exhibited more footshock-induced freezing, especially during juvenile, and emitted more 22 kHz vocalization in response to footshock, which reflects affective state (Zimmerman et al, 2013). A greater vulnerability to stress in MAM rats was also supported by another study that observed larger impairment in PFC plasticity after exposure to stress in MAM rats (Goto and Grace, 2006).

Anxiety-like behaviors in rodent can be measured in many tests, including elevated maze, open field, light-enhanced acoustic startle, novelty suppressed feeding, or light/dark box. One caveat of our studies is that only one behavioral test being used for anxiety-like behaviors is one caveat of these studies. Although our findings on heightened level of anxiety as tested in elevated plus maze was in accordance with other findings of abnormal stress response in MAM rats, novelty suppressed feeding or light/dark box tests could be good candidates to further examine anxiety-like behaviors in MAM- treated rats.

Mechanisms underlying this heightened response to stress are unclear. Given the central role of amygdala in regulation response to stress, we hypothesized it is due to change in amygdala activity. Indeed, we observed a hyperactivity of BLA, which is usually associated with enhance fear and anxiety behavior. In addition, both hyperactivity of BLA and heightened level of anxiety were observed in peripubertal and adult MAM rats. However, what is the mechanism underlying hyperactivity of BLA. BLA is a silent region which is always under strong intrinsic inhibition provided by local interneurons, which makes sure that it will not respond to irrelevant stimuli (LeDoux, 2007). Interneurons that express parvalbumin are fast spiking interneurons that provide a strong inhibition, but we did not see a significant difference on density or number of parvalbumin interneurons in the BLA of adult MAM rats. Further studies looking at other types of interneurons are necessary to investigate if hyperactivity of BLA is attributed to loss of intrinsic inhibition. Another possibility is that hyperactivity of BLA is driven by other brain regions. BLA is extensively interconnected with the hippocampus and PFC. Alterations in both brain regions are important in pathophysiology of schizophrenia and have been observed in rats with MAM model of schizophrenia. Although not studied directly, altered activity of the hippocampus during peripubertal period has been suggested by loss of parvalbumin interneurons

and increased VTA dopamine population activity (Chen et al, 2014; Gill and Grace, 2014). In addition, the hippocampus is important in regulation of both behavioral and physiological response to stress (Herman and Mueller, 2006; McEwen, 2007). Therefore, a disinhibited and arrhythmic hippocampus may result in hyperactivity in the BLA. PFC plays an important role regulating fear and stress response (Maren and Quirk, 2004). Therefore hyperactivity of the BLA might also be attributed to an impaired PFC in MAM rats (Goto and Grace, 2006). Deficits in prefrontal cortical function could limit the ability of this structure to attenuate anxiety and stress responses (Rosenkranz and Grace, 2001).

5.4 TREATMENT OF SCHIZOPHRENIA USING DIAZEPAM

Diazepam (market name: valium) is a widely used anti-anxiety drug in the benzodiazepine family. Diazepam significantly increase the percentage of time spent on the open arms and the number of entries into the open arms of rats on the elevate plus maze test (Pellow et al, 1985). Benzodiazepines have been studied extensively as treatments for schizophrenia. They are more likely to be useful as adjuncts to antipsychotics in treatment for psychosis but their efficacy in schizophrenia is controversial (Wolkowitz and Pickar, 1991). However, in current studies, the time when diazepam was administered is earlier, which corresponds to the adolescent period in humans. During this period, heightened level of anxiety is one of the most obvious phenotypes in these subjects, but psychotic symptoms have not fully emerged yet. Thus alleviating anxiety using diazepam or other treatment, pharmacologically or non-pharmacologically, might be effective methods to prevent transition into psychosis later in life.

5.5 MECHANISMS OF NEURAL OSCILLATIONS

Neural oscillations are a fundamental mechanism for enabling coordinated activity during normal brain function. Oscillations are functionally relevant with a variety of behaviors and cognitions, including sensory perception, attention, working memory (Buzsaki and Draguhn, 2004). These cognitive deficits were all observed in schizophrenia patients (Lewis et al, 2012).

GABAergic interneurons play an important role in generation of oscillations. Pyramidal cells not only project long range to other brain regions, but not form connections with local interneurons. Local interneurons that receive excitatory projection from pyramidal cells exert a powerful control over a population of pyramidal cells through extensive inhibitory innervations. This feed-forward inhibition enables synchronized firing of pyramidal cells. Optogenetic studies have demonstrated that the key role of cortical fast spiking parvalbumin (PV) interneurons in generation of gamma oscillations (Sohal et al, 2009). Indeed, loss of PV interneurons and impairment in gamma oscillations are marked in schizophrenia patients (Volk and Lewis, 2002; Spencer et al, 2004, 2008; Trautner et al, 2006). Similar deficits in oscillatory activities have also been observed in several animal models (Lodge et al, 2009; Gruber et al, 2010; Lanre-Amos and Kocsis, 2010; Sigurdsson et al, 2010). Alterations on oscillatory activities at other frequency bands and synchronization among different brain regions have also been reported in schizophrenia patients (Uhlhaas and Singer, 2010).

However, in the hippocampus, a robust synchronization is centered at theta band (4-8 Hz). Parvalbumin interneurons contribute to theta oscillations in the hippocampus (Wulff et al, 2009). Other types of interneuron may also be involved, e.g. interneurons that express cholecystokinin (CCK) play a complementary role with PV interneurons in hippocampal theta oscillations (Klausbergeret al., 2005). Despite a loss of parvalbumin interneurons in vSub of

MAM rats, observed in current studies and by other researchers (Penschuck et al, 2006; Lodge et al, 2009; Chen et al, 2014; Gill and Grace, 2014), no alteration in hippocampal theta oscillations was found (Lodge et al., 2009, Ewing and Grace, 2013). Probably intact levels of other interneurons help to maintain the level of hippocampal theta oscillation given that overall GABAergic signaling in the hippocampus is unaltered and that the reduction is specifically for PV interneurons.

Very few studies have been done to study mechanism of BLA theta oscillation. In one study, decreases in BLA parvalbumin interneurons, decreased theta power in response to conditioned cues, and decreased fear conditioning were observed in a genetically modified mice (Barkus et al., 2014). These phenotypes may just happen by coincidence, but interestingly it is in contrast to what we have observed in our studies- we did not see decreases in PV interneuron in the BLA of MAM rats but instead a potential trend of increases, and we saw increased theta power in response to conditioned cues, as well as a trend for an increase in fear conditioning.

Another study examining synchronized firing of BLA interneurons with hippocampal theta oscillations found that firing of PV+ basket cells does not tune with noxious stimuli or with hippocampal theta, but interneurons that target dendrites and express calbindin are tuned to hippocampal theta, which raises the question of whether interneurons in the BLA may play different roles from those in cortical areas (Bienvenu et al., 2012).

5.6 HYPOTHESIZED DEVELOPMENT OF SCHIZOPHRENIA-LIKE PHENOTYPES IN MAM-E17 MODELS

MAM administration on E17 is thought to disrupt cell division. Therefore, it has a large impact on the hippocampus, which is actively developing during this time. In addition to disrupting normal brain development, it may work via a genetic or epigenetic mechanism, supported by a recent finding that some MAM offspring of second and third generations still exhibit symptoms related with schizophrenia (Perez et al, 2015). However, the cellular mechanism of MAM administration is unclear.

Based on our findings and studies on human subjects at risk for schizophrenia (Owens et al, 2005; Yung et al, 2005; Corcoran et al, 2012; DeVylder et al, 2013), hyperresponsivity to stress contributes to the development of schizophrenia. In MAM-treated rats, prenatal insult disrupts brain development, and probably alters genetic expression in the brain, both of which contribute to heightened levels of anxiety and hyperresponsivity to stress observed in MAM rats in the peripubertal period. Hyperactivity in both BLA and the hippocampus could contribute to these behavioral alterations. With higher anxiety, MAM rats are more vulnerable to everyday stress. And more stressful experience perceived in MAM rats will further damage brain regions sensitive to the deteriorating effect of stress, e.g. the hippocampus, especially parvalbumin interneurons in the hippocampus. Ultimately, when these rats reach adulthood, they have a full blowout of schizophrenia-like symptoms. However, with early interventions to alleviate anxiety/ hyperresponsivity to stress (e.g. peripubertal diazepam administration), further damage to the hippocampous and other brain regions by stress will be attenuated (e.g. the loss of parvalbumin interneurons in the hippocampus was attenuated). Therefore, the system could be maintained

under a threshold so that the cascade that leads to ultimate blowout of those symptoms will not be triggered.

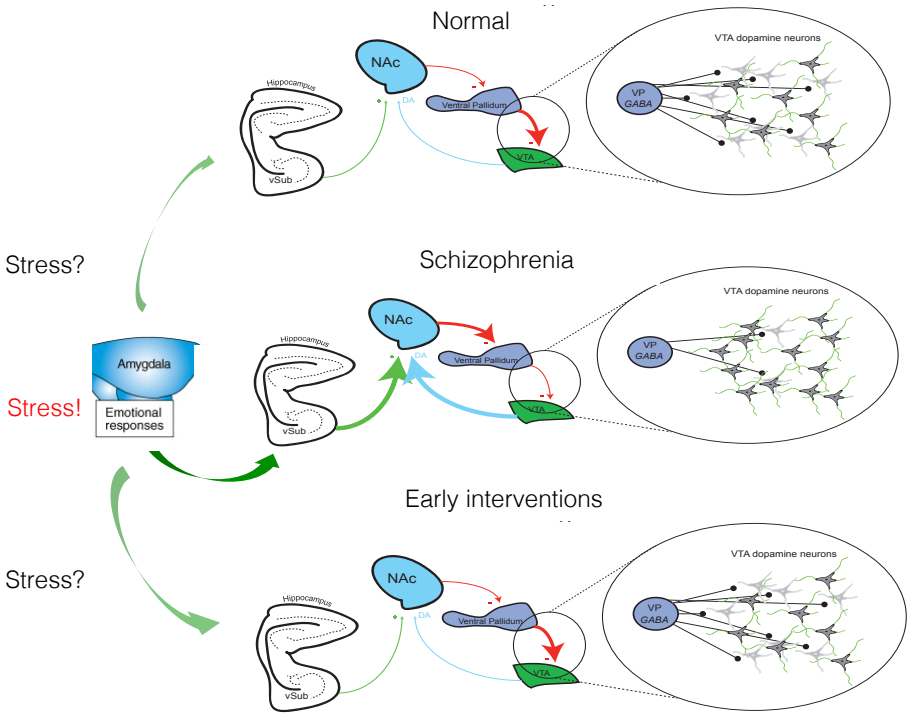

Figure 12. Hypothesized development of schizophrenia-like phenotypes in MAM-E17 models.

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